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FEVER AND HODGKIN'S DISEASE:
STUDIES ON PYROGEN RELEASE IN VITRO
BY LYMPHOID TISSUE

MARVIN MICAH CHASSIN

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FEVER AND HODGKIN'S DISEASE:
STUDIES ON PYROGEN RELEASE IN VITRO
BY LYMPHOID TISSUE

by
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A thesis submitted to the faculty of the
School of Medicine, Yale University
in partial fulfillment of the
requirements for the degree of
Doctor of Medicine

Department of Internal Medicine
Yale University School of Medicine
New Haven, Connecticut
1973

Dedicated With Love
to Barbara

ACKNOWLEDGMENTS

I wish to express my appreciation to:

My advisor, Dr. Phyllis Bodel, for her inspiration,
encouragement and guidance in the formulation,
performance and completion of this work;

Dr. Elisha Atkins for his invaluable suggestions
and his unequalled enthusiasm for the
adventure of biological research;

Mrs. Judith Smillie and Mrs. Lorraine Francis
for their invaluable technical assistance;

the following surgeons who obtained the tissue
used in these experiments: Drs. S. Brodoff,
E. Cahow, I. Goldenberg, M. Hayes, S. Nuland,
L. Pickett, N. Passarelli, C.F. Roe, R. Selzer,
B. Siegal, R. Toloukian, and A. Toole;

My wife, Barbara, without whose assistance,
encouragement, support, and love, this work
could not have been achieved.

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INTRODUCTION

Fever, the abnormal elevation of central body temperature, is a common accompaniment of many clinically recognized pathologic states. Among these are infections, neoplastic diseases, connective tissue disorders, vascular accidents, hemolytic states, drug or chemical reactions, and metabolic abnormalities. (11). A mechanism for fever production, involving endogenous pyrogen production and release by host cells in response to an appropriate stimulus, has been proposed on the basis of evidence derived from many experimental situations, induced primarily in laboratory animals, occasionally in man (4). While it has been assumed that the same mechanism is involved in the production of fever in the above mentioned clinical states, direct evidence for this assumption is not extensive. The following work is an attempt to relate the fever of Hodgkin's Disease, a disease often characterized by fever, to the proposed mechanism of fever production involving endogenous pyrogen.

Fever and Neoplastic Disease

Fevers often complicate the course of patients with neoplastic diseases (22, 27, 28). However, while the overall incidence of fever in cancer patients is high, the significance of the fever to the patient's course is often low (27). Various series reviewed by Boggs and Frei (22) and Browder, et. al., (28) covering patients with various, primarily internal, solid tumors, showed the incidence of fever in these patients to be over 40%. Most of these fevers, however, were isolated rises in temperature. In only 3% of Briggs' patients, for instance, was the fever deemed to be a prominent feature of the patient's course (27). Furthermore, the fever, in most cases, could be ascribed to a coincident infection, often secondary to obstruction caused by the tumor. Browder, et. al., in carefully examining their series of febrile cancer patients, concluded that fever in the absence of infection or obstruction was indeed rare. They explained the earlier reports of high fever incidence among these patients by the high incidence of infection and obstruction amongst patients with these cancers, particularly those with cancer of the lung or of the gastrointestinal tract. In their series, they were able to find only 5.4% of 351 patients who had fever related to their disease, without infection or obstruction (28). For the most part, then, the problem of explaining fever in neoplastic disease is

really a problem of explaining fever in infection.

There are, however, certain exceptions. One is disease metastatic to the liver. In Browder's group, the incidence of fever in the absence of demonstrable infection was two and one half times greater among patients with hepatic metastases (28). Earlier, Fenster and Klatskin had found that 23% of their febrile patients with carcinoma or sarcoma metastatic to the liver had fever unassociated with infection (40).

Other exceptions are those certain neoplasms whose presence is often early associated with fever, and often without associated infection, obstruction or hepatic metastasis. Among these are renal cell carcinoma, (14, 24, 31, 44, 62, 99), and Hodgkin's Disease (22, 65, 90, 91).

In 1929, Creevy reported two cases of fever associated with malignant renal tumors, one in which there was no metastatic disease, and concluded that, since the fever could not be related to necrosis in the tumor nor to metastasis, it was due to a product of the abnormal metabolism of the tumor tissue (35). Since that time, there have been reports placing the incidence of fever in patients with this tumor at between ten and twenty percent (31, 24, 44). (In a review of 273 cases Berger and Sinkoff (14) put the incidence at 16%.) The fever is often found in the absence of extensive hemorrhage

or necrosis in the tumor, in the absence of metastatic disease, without relation to the size of the tumor or the extent of local invasion, (62) and, as can be attributed by the frequency with which patients with this tumor are included amongst series of patients with fever of unknown etiology (23, 24, 41, 51, 61, 79, 83, 99), it is often the presenting symptom of the disease. Moreover, the fever is so predictably relieved by removal of the tumor, that persistence of the fever after operation for resection of the primary, or its recurrence at some later date, suggest the presence of undiscovered metastatic disease (74).

The fever associated with Hodgkin's Disease has been a particularly intriguing aspect of the clinical picture of that disease for over 100 years. Among the first patients described by Hodgkin in 1832 was a 25-year-old woman who had two intermittent attacks of fever (52). In 1858, shortly after the rediscovery of Hodgkin's work by Wilke, Wunderlich described a patient in whom there were periodic temperature elevations coincident with enlargement of the lymph nodes and spleen (101). In 1870, Murchison described a six-year-old with a two and one half year course of periodic febrile episodes, which also were associated with nodal enlargement (32). In 1879, Gowers gathered 113 cases of Hodgkin's Disease, in 40 of whom attention had been paid to the temperature (45).

Twenty-seven had fever which could be related to the clinically determined activity of the disease, and only thirteen were afebrile or had fever attributable to an intercurrent infection. He was the first to draw attention to that unusual and interesting pattern of relapsing fever which was to be frequently reported in the ensuing years and to become widely known as Pel-Ebstein-Murchison fever. In this pattern, several days of fever which gradually builds in degree, then gradually recedes by lysis, not by crisis, is followed by a period of normal or subnormal temperatures. He also described two other, more common, patterns of fever in these patients-continuous, with minimal diurnal variation, and intermittent, with evening exacerbations. There followed many reports of the more interesting, but rarer, relapsing pattern. Osler described a case with intervals of fever associated with fluctuating lymph node size, increasing with the degree of pyrexia, decreasing during the afebrile periods (67). Pel earned his eponym with a description of a 25-year-old man who had five episodes of fever lasting between ten and 31 days, associated with increasing splenic size (78). In 1887, Ebstein reported a case, followed for 238 days, during which there were nine attacks of fever, of two weeks duration, spaced by afebrile intervals, of ten or eleven days (38). In 1904,

Taylor reported three more cases with the relapsing type of fever, including one gentleman whom he had followed through five febrile episodes of seven to thirty days duration with afebrile intervals of six to seventeen days, who then remained afebrile for four months before his eventual death (96). He was the first to point out that of the three patterns of fever in Hodgkin's Disease patients as described by Gowers--continuous, intermittent, and relapsing, the last was most rare, but when present, the most pathognomonic of the disease. In 1911, MacNalty gathered together 27 cases of relapsing fever in Hodgkin's Disease reported in the literature and added five of his own, using the eponym Pel-Ebstein-Murchison fever to describe them (67).

More recent studies have been concerned with the significance of fever in Hodgkin's Disease patients. Lobell, et.al, found that 60% of Hodgkin's Disease patients have fever (defined as a single reading greater than 103°F or a temperature greater than 100.4° for three of five consecutive days) at some point during the course of their disease (65). Looking at the biased sample of hospitalized patients, Boggs and Frei found that Hodgkin's Disease patients have the highest rate of febrile days to total hospitalized days amongst patients with neoplastic diseases, and that the fever in these patients was more likely to be unassociated with demonstrable infection than in patients with other lymphomas, leukemias, or solid tumors (22).

In Lobell's study, fever was classified as infection-related or disease-related. One half the patients followed in their clinic had disease-related fever at some point in their course, while only 13% had fever related to documented infection. Fever present at the time of diagnosis (one third of their patients were in this group) was almost exclusively disease-related. Even late in the course of the disease, however, a patient with fever was more likely to have the fever of Hodgkin's disease than to have fever secondary to infection.

They also found that fever is a bad prognostic indicator independent of certain other bad prognostic signs, that the fever thought to be disease-related could not be distinguished from the fever of infection by the character of the temperature chart alone, and that Pel-Ebstein-Murchison fever is indeed rare, being present in only one of their 137 patients.

Mechanism of Fever

Fever is the abnormal elevation of central body temperature. It may be brought about by direct activation of peripheral mechanisms that increase heat production or reduce heat loss, but more often is the result of a central alteration of body temperature regulation (32, 33). Body temperature is normally maintained relatively constant by regulation of heat production and heat loss. This is thought to be a function of certain centers in the hypothalamus which act in response to peripheral and central thermoreceptor input. During fever, regulation of body temperature appears to proceed in a normal fashion, but at an altered set point (4, 33, 100). The mechanism by which a new set point is established is not entirely known, but it appears, in many animal models, that a common pathway is the release of endogenous pyrogen (EP) by cells peripherally, and its action at some point centrally (4).

Evidence for this pathway consists of demonstration of EP production by cells from the periphery, demonstration of circulating EP, and demonstration of central effect. In many experimental systems various exogenous and endogenous pyrogens including Gram negative bacterial endotoxins, gram positive bacteria, viruses, antigen-antibody complexes, and antigens have been shown to effect the production and release of an endogenous pyrogen from cells capable of its

production (4, 6, 30, 85, 94). Circulating EP has been found in many of these systems, including induced streptococcal and pneumococcal infections, intravenous injections of virus, intravenous injection of endotoxin, and intravenous injection of antigen or antigen-antibody complexes (4, 9, 46, 71, 85). Centrally, EP has been shown to induce fever upon anterior intrathecal injection of one percent of the amount required to produce a similar effect by IV injection, and to produce changes in the firing pattern of thermosensitive neurons in this area similar to those seen upon local cooling (4, 100).

It is presently unclear whether endogenous pyrogen represents the final common pathway or merely a common pathway for elevation of the set point by many varied stimuli. Its effect at the hypothalamus may very likely be mediated locally by other intra or extra cellular messengers, as is the effect of hormones at their target tissues (100).

To summarize, the postulated mechanism of fever production involves the interaction of an appropriate stimulus with a cell capable of production and release of endogenous pyrogen leading to the release of this mediator. EP travel via the bloodstream to the anterior hypothalamus where, possibly via the release of other substances, it causes a change in the pattern of neuron discharge which effects net heat accumulation, elevation of central body temperature,

and maintenance of this new temperature level.

Endogenous Pyrogen

Endogenous pyrogen is a protein-containing substance of molecular weight between ten and twenty thousand (21, 63, 73). It has been shown to be produced by a number of cell types. Blood leukocytes were early recognized as a source of EP. In 1948, Beeson first isolated a pyrogen-producing substance from rabbit granulocytes and subsequently, together with Bennett, in the classic article in the field, distinguished this pyrogen from bacterial endotoxin (11, 12, 13). They were unable to isolate EP from other cells. Later, Gerbrandy, Cranston, and Snell demonstrated that pyrogen released human blood was a variable, dependent on the granulocyte content of that blood (42).

However, situations were recognized in which EP release by cells other than polymorphonuclear leukocytes had to be postulated. Included were fever in agranulocytosis and in inflammatory processes characterized predominantly by a mononuclear response such as the granulomatous diseases, including Hodgkin's disease (13).

It has since been shown that cells other than polymorphonuclear leukocytes are endogenous pyrogen producers. Mononuclear cells from rabbit lung, and from experimentally produced sterile exudates, produce EP when suitably activated in vitro (5, 49). Also human blood monocytes, from normal

individuals or patients with monocytosis (19), and rabbit hepatic Kupfer cells (36) have been shown to release EP in vitro when activated. The presence of pyrogen in tissue monocytes may be the explanation for the pyrogenic activity of saline extracts from normal rabbit tissues (10, 93a). Circulating human eosinophils have recently been shown to release EP when activated in vitro by phagocytosis or bacterial endotoxin (69). Studies by Snell and Atkins, by Root, et. al., and by Kammermeyer, et. al., have failed to demonstrate EP release by circulating, lymph node, or cultured lymphocytes (4, 56, 84). Lymphocytes have, however, been shown to produce a substance capable of provoking EP release from competent cells (7).

Endogenous pyrogen production by PMN leukocytes only occurs following cell stimulation (17, 74, 55). Although the exudate granulocyte has been found to be spontaneously active, an activator has been isolated from the exudate fluid which is presumably responsible (70). Activation of PMN leukocytes may follow stimulation by endotoxin, phagocytosis, antigen-antibody complexes in the presence of complement, or, perhaps, by lymphokine mediator (3, 7, 34, 42, 74, 94, 100). An early, essential response of the cell to stimulation is new RNA and protein synthesis. Measurable EP release begins at about two hours after activation and continues for only 12-16 hours. Release of EP may be

suppressed by agents that block protein synthesis or glycolytic pathways only if such agents are added during the initial two-hour period after the activation (17, 74).

Blood and tissue monocytes appear to behave differently in several respects (5, 15, 19). They release 4-10 times the amount of pyrogen for the same stimulus as do PMN leukocytes and they continue pyrogen release longer after activation, perhaps 48-72 hours. Also, throughout this period, pyrogen production by these cells can be suppressed by inhibitors of protein synthesis.

Mechanism of Hypersensitivity Fever

Clinical association between fever and hypersensitivity reaction has been long recognized, as in conditions such as serum sickness and drug reactions (4). Experimentally, injection of an antigen into a sensitized animal will result in fever (71). Serum antibody appears to be essential to this response. Wolff's group has suggested that it is the antigen-antibody complex which is the stimulus to endogenous pyrogen production in this situation, as antigen-antibody complexes prepared in vitro produced fever when injected into unsensitized animals (71, 85). Attempts to produce EP release by blood cells in vitro using antigen-antibody complexes has usually been unsuccessful. Atkins failed to induce EP production using complexes of bovine gamma globulin

(BGG) and rabbit anti-BGG prepared in antigen excess (7). In other experiments, when the benzyl penicilloyl nucleus of penicillin was incubated with serum and cells from sensitized animals, Atkins and Chusid were able to demonstrate EP release. The exact nature of this hypersensitivity reaction was not defined, however (30).

Fever production has also been studied in systems of delayed hypersensitivity. Injection of Old Tuberculin causes fever in tuberculin sensitive rabbits, and this response can be transferred to non-sensitized rabbits by mononuclear cells as well as by serum (4, 50, 50a). Blood leukocytes and tissue macrophages from tuberculin-sensitized animals release EP upon in vitro incubation with antigen (5, 8). This suggests that antigen has a direct effect upon polymorphonuclear and mononuclear cells, inducing EP release.

Recently, using another model Atkins, et. al, demonstrated a role for lymphocytes as mediators leading to endogenous pyrogen release in a system of delayed hypersensitivity. Rabbits were immunized to dinitrophenol (DNP)-conjugated bovine serum albumin, to DNP-bovine gamma globulin, or to human serum albumin. Upon incubation of lymph node lymphocytes obtained from these sensitized animals with the appropriate antigen, a substance, perhaps a lymphokine was released which, while not itself pyrogenic, induced the release of EP from normal blood leukocytes (3, 7).

Clinical Fever and Endogenous Pyrogens

Although the mechanisms of EP production discussed above have been postulated to account for clinical fever in man, there is little experimental evidence for this hypothesis (4). Such evidence ought to include (1.) demonstration of endogenous pyrogen production by human cells in response to appropriate stimuli, (2.) isolation of EP from patients with diseases characterized by fever, (3.) detection of EP in the circulation in association with fever, and (4.) induction of fever by EP administration.

Several human cell types have been shown to produce endogenous pyrogens in vitro. Polymorphonuclear leukocytes, blood monocytes, eosinophils, and spleen and lymph node mononuclear cells are all capable of EP release if suitably stimulated by phagocytosis (16, 19, 69, 74). Spleen and lymph node cells, predominantly mononuclear, are occasionally "spontaneously" active in vitro, unlike the other cell types which require activation (16). In addition, cell suspensions from renal cell carcinomas have been found, by Bodel, to be capable of EP release during in vitro incubation (15).

Several studies have demonstrated the presence of an endogenous pyrogen in fluids obtained from patients, although not always febrile ones. In 1962, Snell reported the presence of a pyrogen, with properties characteristic of EP, in exudate fluid from malignant, bacterial, and tuberculous

effusions (93). A similar substance was not present in transudates from patients in congestive heart failure. However, there was no correlation between EP isolation and the presence of fever in the patient. Bodel and Hollingsworth reported that a pyrogen, active in rabbits, was present in the joint fluids of patients with such diseases as rheumatoid arthritis, acute rheumatic fever, pseudogout, Reiter's syndrome, and metastatic carcinoma (20). Sokal and Shimaoka have demonstrated a pyrogen in the urine of febrile patients with Hodgkin's disease and another febrile patient, not present in the urine of normal controls (95). Finally, Rawlins, et. al, found a similar pyrogen in renal carcinoma tissue (82).

The search for circulating endogenous pyrogen in clinical febrile states has been uniformly unsuccessful. Snell was unable to demonstrate circulating EP by re-infusion of plasma taken from patient febrile with various infections (92). Bouruncle and Doan tried reinfusing 500 ml. of whole blood from patients febrile to 103° F with periodic fever, and, similarly, failed to induce fever in these same patients, when they had become afebrile (25). Sokal and Shimaoka were unable to produce fever by reinfusion of plasma obtained from febrile Hodgkin's disease patients (95). Cranston, using malaria patients, was able to invoke fever on reinfusion of the cellular elements,

but not the plasma; this result seems more likely due to the host response to altered cells, or to the parasite, than to a preformed pyrogen (32).

Since Bodel and Atkins have shown that human endogenous pyrogen can be assayed in rabbits (18), the necessity for reinfusion studies has been eliminated, but the results have not changed. Greisman, et.al, using a rabbit assay that permitted pyrogen testing up to 20 ml. of human plasma in rabbits, after absorbing out the hemagglutinins, were unable to demonstrate a pyrogen in plasma drawn from febrile (104° - 105°F) patients with Rocky Mountain Spotted Fever (147).

Even when febrile reactions have been experimentally induced in humans, circulating EP has not been found. Until recently there was only one encouraging result reported. Whole blood taken from a volunteer at the height of a fever (38.2°C) caused by an injection of heat killed E. coli, induced an early (20 minute onset) fever in a second subject of the same blood group. Subsequently attempts have been made to find circulating EP by cross infusion or reinfusion of cells or plasma from subjects febrile due to EP infusion, 11-keto-pregnanolone infusion and S. typhosa endotoxin injection (43, 47, 59, 81). These all have failed.

Most recently, Greisman and Hornick have produced an early (45 minute onset, 90 minute peak) temperature elevation by infusion of 750 ml. of plasma pooled from three donors made febrile to 102°F by *S. typhosa* endotoxin (47).

While the proposed mechanism for fever production via endogenous pyrogen release requires that EP be present, at some time in circulating blood, the failure to demonstrate it there does not necessarily imply that it is never present. Perhaps the failure to demonstrate it in these many experimental and clinical situations relates to the amount of pyrogen present in the fluid sampled, its distribution in the sample with respect to protein binding, the time of sampling with respect to fever present in the donor, or the failure of the assay method to reproduce a physiologic method of presentation of the pyrogen to its receptor at the hypothalamus.

Cranston's group showed that endogenous pyrogen produces fever in man when they infused EP prepared by endotoxin stimulation of blood cells (34, 42, 81).

Fever in Malignant Disease

The relation between the fever of malignant disease and the proposed mechanism of fever discussed above, is largely unknown. Clinically, most fevers in cancer patients

are related to infection (28). Thus inflammation, necrosis, and immune reaction may all be present and all of these processes have been experimentally linked to EP production (4, 53). However, there are some tumors, discussed previously, which appear to be more frequently associated with fever in the absence of demonstrable infection. These tumors, which include renal cell carcinoma and Hodgkin's disease have received particular attention because of the possibility that fever in these states might be definitively associated with endogenous pyrogen.

Rawlins, et al, detected an endogenous pyrogen in tumor extracts from two febrile patients with renal cell carcinoma (82). They found no EP in normal kidney, in partially necrotic tumor tissue from one afebrile patient with renal cell carcinoma, or produced by circulating leukocytes from the febrile patients. Bodel has found that cells from renal carcinomas, which have not been previously irradiated, spontaneously release EP upon incubation in tissue culture medium containing patient serum, whereas cells from irradiated tumors, even when necrotic, and cells from adjacent normal kidney produce little or none (15). This in vitro EP release has not, however, correlated with the presence of fever in the patient. It appears that there are other factors beyond capacity for EP release which determine whether the capacity for EP release becomes manifest as

fever in the patient. Activation may be one important factor, as the cells may require a specific stimulating event in vivo. Other factors may be rate of absorption into the circulation, suppression of EP production in vivo, inactivation of the pyrogen itself in the circulation or at the target tissue, or modification of the target tissue's response to the EP stimulation.

Since renal cell carcinoma has been widely implicated in other paraneoplastic syndromes related to the elaboration of a protein or peptide by tumor tissue, it may be that the tumor cells themselves are synthesizing EP. However, the pyrogen may be from inflammatory cells present in the tissue, although, if such were the case, one would expect EP production from cells in the previously irradiated kidney tumor as well.

In one case of neuroblastoma, which is also frequently associated with fever (39), Chassin and Bodel were unable to demonstrate EP release after 72 hour incubation of tumor cells from a febrile patient (19a). However, this tumor, which, like the pheochromocytoma, makes vasoactive amines, may cause fever by vasoconstriction and decreased heat loss, thus altering the efferent, rather than the afferent limb of the temperature regulatory process.

The first observation concerning an endogenous pyrogen in Hodgkin's disease patients was made by Sokal and Shimaoka in 1967 (95). Like other investigators trying to demonstrate

circulating EP in febrile patients they were unable to demonstrate EP in the plasma of febrile Hodgkin's disease patients. They had attempted this by reinfusion of autologous plasma to these patients when afebrile. However, in examining urine concentrates, they found a pyrogen capable of causing fever in rabbits. Concentrates representing 0.03 - 1.0% of a twenty four hour urine collection from six of seven febrile patients caused fever in rabbits, whereas none of four concentrates from normal controls caused fever. They did not examine the urine of afebrile Hodgkin's patients. A concentrate from a febrile, non-Hodgkin's diseased patient behaved similarly to that from the febrile Hodgkin's patients. In each case the fever curve obtained was characteristic of that caused by EP, with a rapid onset after injection, and a peak at one hour. There was suppression but not elimination of the fever when the material was tested in endotoxin-tolerant rabbits; repeated injections of the pyrogen daily for one month caused no tolerance.

In 1971, Young and Hodas described, in an abstract, the isolation of a protein from the urine of febrile Hodgkin's disease patients which was not present in the urine of afebrile patients with the disease (101). This material behaves, under electrophoresis, like a protein which is present in normal cerebrospinal fluid and in the

plasma of febrile Hodgkin's patients. It has a molecular weight of 15,000 similar to that described for endogenous pyrogen (21, 63, 73), but also has an isoelectric point of 10, which is unlike any described for EP. The isoelectric point for human EP is about 7.0 (73). It is unclear whether this protein can be related to EP, and its significance remains obscure in the absence of any demonstration of pyrogenicity.

Most recently, Bodel has examined the capacity of various tissues from patients with Hodgkin's disease to produce endogenous pyrogen (15, 16). Blood leukocytes from these patients were indistinguishable from normal donor's leukocytes in that they did not spontaneously produce pyrogen, but invariably did so when activated by phagocytosis. However, lymph node and spleen cells from patients with lymphomas released EP upon in vitro incubation in tissue culture medium. Spleen cells from patients with non-malignant splenomegaly did not release EP spontaneously, but did so in response to phagocytosis. Since production of EP continued for 48-72 hours in lymph node suspensions and since this production was sensitive to inhibition by puromycin, mononuclear cells appear to be responsible for pyrogen production in this tissue. In spleen cells, the production of pyrogen did not correlate with percentage of granulocytes in the preparation;

however since cycloheximide was only partially inhibitory, both polymorphonuclear and mononuclear leukocytes may contribute to EP release by this tissue. The production of EP did not correlate with presence of clinical fever in the patient, nor with pathological evidence of tissue involvement by the disease.

Hypersensitivity and the Pathogenesis of Hodgkin's Disease

The histology of Hodgkin's disease is such that there has long been question as to whether the process is neoplastic or granulomatous (58). The Jackson and Parker classification graded the histology from paraganuloma to sarcoma with the prognosis being better for the paraganulomatous type than for the more frankly malignant sarcomatous (54). The Lukes and Butler classification takes account of the different histories of the patients with different histologic lesions in its progression from a lymphocyte predominance type with a better prognosis to the lymphocyte depletion type with a worse one (66). It appears that the lymphocytes may represent a reactive element in the tissue whose presence can be correlated with a longer survival. The cells predominating in the lymphocyte depleted lesion, the reticulum cells and the Reed-Sternberg cells, appear to be proliferative elements, perhaps the neoplastic cell line whose predominance is correlated with a poorer survival. The suggestion is that the disease represents a mixed reactive-proliferative state, that the reactive element is the earlier one, and that it in some way may lead to or be replaced by the later proliferative state.

These observations have suggested to many a resemblance

between Hodgkin's disease and a graft versus host reaction (58, 97, 29). Smithers and Kaplan, in 1959, first proposed that the proliferative elements were lymphocytes that had lost some of their antigenic determinants, and therefore recognized normal host cells as foreign, leading to a reaction against the host and their own proliferation (58). Tyler, in 1960, made a similar proposal (97). Since then Schwartz and his colleagues, as well as others, have provided a strong experimental model for lymphomas resulting from chronic graft versus host disease (2, 64, 87, 88, 89).

The requirements for producing the disease are an immunocompetent graft, a host antigen recognized as foreign, and an immunoincompetent host. In experimental models, the first is provided by a parental spleen cell implantation, the second by the antigens of the other parent strain expressed by the F_1 hybrid, and the third by a tolerant F_1 hybrid or an immunosuppressed adult animal. The result is an early lymphoid reaction and late lymphocyte depletion, repopulation of the area by plasma cells and reticulum cells, and, in a certain percentage of cases, a proliferative reticulum cell or plasma cell population. There is also splenomegaly and lymphadenopathy clinically, impaired systemic immune responsiveness, and, in some models, autoimmune hemolytic anemias or immune complex glomerulonephritides (68).

An interesting finding, by the Schwartz group, is that the proliferative cells are not of the immunocompetent parent line, but of the immunoincompetent host line (89). Apparently the host cells are stimulated by the immunocompetent cells, or by the damage they cause, to undergo neoplastic transformation. This may be by mutation, such as if the products of lymphocyte breakdown cause mutation that leads to a loss of proliferative regulation. It also may be that the immunodepression caused by the graft versus host disease allows the proliferation of cells containing a viral oncogene. These cells could be of host origin, but would also perhaps be recognizable by a viral-associated antigen which they might display.

Recently, Order, et al, demonstrated by immunofluorescence such a tumor-associated antigen in Hodgkin's disease tissue, not present in adjacent normal tissue (75, 77). Also, using immunoelectrophoresis they were able to demonstrate two antigens common to 18 of 19 Hodgkin's disease spleens, two of four suspicious spleens, and four of seven pathologically negative spleens, but in increased concentration in the positive spleens (75). This further piece of evidence suggested to them a hypothesis which they proposed for the pathogenesis of Hodgkin's disease (76). In it, host T cells are infected by a virus leading to a changed

surface antigen (26) - perhaps the tumor associated antigen which they have found. If the transformed cell also manages to maintain host tolerance, a graft versus host reaction might ensue, leading first to lymphocyte reaction and, in time, to lymphocyte depletion with repopulation by plasma cells and proliferating reticulum cells and their end stage Reed-Sternberg cells. Because of T cell involvement in the graft versus host reaction, there would be systemic anergy, a condition frequently associated with active Hodgkin's disease (1).

If their hypothesis is correct, then a further consequence would be that in the presence of an ongoing cell mediated immune reaction, such as the graft versus host reaction proposed here, at least for the early part of the pathogenesis, there would be T cell blast transformation and lymphokine production. If one of these lymphokines is a factor which activates cells competent to produce endogenous pyrogen to do so, as proposed by Atkins (7), then we might expect to demonstrate such a lymphokine in lymphoid tissue from patients with Hodgkin's disease.

OBJECTIVES

The objective of the research discussed below is to investigate the release of pyrogen in vitro by cells from patients with Hodgkin's disease. The three separate aims of the three studies which make up this work are outlined below, based on information discussed in the introduction.

I. Comparison of spontaneous pyrogen release in vitro by lymphoid tissue from patients with Hodgkin's disease with that by lymphoid tissue from patients without Hodgkin's disease.

As noted, endogenous pyrogen release is a well established pathway in the mechanism of fever production in a number of experimental models, but evidence for this pathway in clinical fever is scanty (4). Second, fever commonly accompanies Hodgkin's disease in the absence of demonstrable infection (65). Third, EP release in vitro has been demonstrated for cells from another tumor commonly associated with fever - renal cell carcinoma, and a pyrogen has been found in the tissue of this tumor (15, 82). Finally, EP release in vitro has been demonstrated for lymphoid tissue from patients with malignant lymphomas (16).

In view of this, the first objective of these experiments is to compare EP release in vitro by cells from lymphoid

tissue of patients with Hodgkin's disease with that by cells from lymphoid tissue of patients with other disorders.

II. Investigation of the effect of serum on pyrogen release by lymphoid tissue in vitro.

The lack of correlation between demonstrable pyrogen release by tissues in vitro and the presence of clinical fever in the patient has been noted above (15, 16). One explanation for this is that the cells have not been activated in vivo to release EP. Alternatively certain factors may be acting in vivo to suppress EP production and release. In order to investigate the latter possibility, the effect of different concentrations of sera upon in vitro EP release was examined.

III. Studies of the action of lymphocytes on induction of pyrogen release by blood leukocytes.

As discussed above, Hodgkin's disease may represent a proliferative response to a foreign antigen or to a self antigen which has been recognized as foreign by abnormal lymphocytes (76, 58, 97). Also, sensitized lymphocytes have been shown to release a substance, perhaps a lymphokine, which may activate normal leukocytes to produce endogenous pyrogen, when these lymphocytes are themselves activated by the sensitizing antigen (3, 7). Release of such a

substance by activated or altered lymphocytes in spleen and lymph nodes from patients with Hodgkin's disease could explain the "spontaneous" release of pyrogen characteristic of these tissues, through interaction of these immunocompetent cells with tissue polymorphonuclear or mononuclear cells capable of endogenous pyrogen production. To examine this hypothesis, lymphocytes from spleen and lymph node tissue were incubated with blood leukocytes in an attempt to induce a pyrogen release.

MATERIALS AND METHODS

I. Sterile pyrogen free technique. All materials, glassware, and reagents used in these experiments were sterile and pyrogen free. Metalware, glassware, and glass wood were sterilized in hot air ovens at 180° C for 2 hours. Solutions were autoclaved at 15 p.s.i. for 90 minutes. Aliquots were incubated in thioglycollate broth and tested for pyrogenicity in rabbits. Reagents not prepared in this laboratory were pyrogen tested before use, or were obtained sterile and pyrogen free.

II. Reagents. Tissue culture medium (TCM). Auto POW Eagle minimal essential medium (Flow Laboratories, Rockville, Maryland) was prepared as follows: 9.39 grams Auto POW medium was made to one liter with sterile water. The solution was autoclaved 20 minutes at 15 p.s.i. pH was adjusted to 7.4 by the addition of sodium bicarbonate solution, and penicillin-streptomycin (Grand Island Biological, Grand Island, N.Y.) was added to 5 units penicillin and 5 micrograms streptomycin per ml. of tissue culture medium. Just before use, heparin (Liquemin Sodium, Organon, W. Orange, New Jersey) was added to tissue culture medium to a final concentration of 10 units per ml.

Modified Krebs-Ringer-Phosphate buffer (KRP buffer). A 1.43% Na_2HPO_4 solution was filtered and adjusted to pH 7.4 with HCl. A base prepared of 40 ml. of 1.15% KCl, 6ml. of 1.22% CaCl_2 and



2 ml. 3.82% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in sufficient saline to bring the total volume to one liter was autoclaved for 90 minutes. 105 parts base and 20 parts phosphate solution and 10 units/ml. heparin were combined just before use.

Human serum albumin. A 25% salt poor albumin solution (American National Red Cross) was added to TCM or to KRP buffer as described below, for the isolation of lymphocytes by the column procedure.

Heat killed Staphylococcus albus. A strain of *S. albus* was maintained on agar. Cells from an 18 hour broth culture of the bacteria were washed with saline, resuspended in saline and autoclaved for 20 minutes. They were stored at -18°C in a suspension of approximately 5×10^9 bacterial cells/ml. estimated by comparison of optical density with a standard curve.

III. Pyrogen Assay. Pyrogen testing was carried out in adult New Zealand White rabbits (Montowese Rabbit Farms, North Haven, Conn.). Animals were boxed for nine hour intervals in a constant temperature room (18.3°C) and temperatures were measured at 15 minute intervals with Telethermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio) or continuously with automatic temperature recording equipment (Rustrak Gulton Industries, Manchester, New Hampshire) via rectal thermoprobe. Rabbits were not

used if a stable baseline temperature was not present for 30 minutes prior to each injection. Each rabbit was used for only 5 consecutive calendar days to prevent interference with the assay due to hypersensitivity to human serum constituents.

"Dose" (see below) refers to the number of cells incubated in the supernate to be assayed. Supernates to be assayed were injected via the ear vein. In most experiments the same rabbits received both control and experimental supernates, and the same supernate was injected into more than one rabbit, whenever sufficient material was available. The maximum temperature elevation occurring between 30 and 75 minutes after injection was charted.

IV. Preparation of tissues. Spleens and lymph nodes were obtained in saline directly from the sterile field at operation. The tissues were weighed, then minced in tissue culture medium, and the spleens additionally screened through wire mesh. The suspensions were cultured in thioglycollate broth, a free cell count was determined by coulter electronic particle counter (Coulter Electronics, Hialeah, Fla.) and a cover glass smear was made to be stained with Wright's stain for differential count.

V. Preparation of normal leukocytes. Venous blood was withdrawn into a heparinized (10u/ml) syringe from normal

volunteers, or, in some cases from patients. It was allowed to sediment for one hour, after which time the leukocyte rich plasma was removed and centrifuged at 500g for 15 minutes. The cells were resuspended in TCM (with heparin 10u/ml) counted by means of a coulter particle counter, and added to the incubation flasks to provide $3-7 \times 10^7$ cells per white cell dose. A cover slip slide differential was performed from blood smears after staining with Wright's stain.

VI. Column separation of lymphocytes. Size #20 glass columns (Kontes Glass Co., Vineland, N.J.) were filled with glasswool under saline using sterile techniques. The eluate saline was cultured. A solution of modified Krebs-Ringer-Phosphates buffer containing heparin (10u/ml) and human serum albumin (0.75-1.5%) was run onto the column and was allowed to equilibrate at 37°C.

A portion of the spleen cell suspension was strained through several layers of sterile gauze and the concentration adjusted to approximately 2×10^7 cells per ml. in TCM with 10u/ml. heparin and 1.5% human serum albumin.

The spleen cell suspension was run onto the column rapidly, allowed to stand 30-40 minutes at 37°C, and eluted with twice the volume of KRP buffer containing heparin and albumin. The eluate was centrifuged at 500g for 15 minutes, and the cell button resuspended in tissue culture medium to be added to the incubation flasks at approximately 2×10^8

cells per dose of lymphocytes. An aliquot was cultured and a Wright's stained cover slip smear was made for differential count.

VII. Incubations.

A. Incubation for spontaneous pyrogen release. Tissue cell suspensions were incubated to provide 0.5-1.8 g. spleen, wet weight ($1.3-7.0 \times 10^8$ free cells) or 50-333 mg. lymph node (wet weight), per dose. Each dose was suspended in 4-5 ml TCM with 10-15% serum, in an Erlenmeyer flask. The flasks were flushed with 5% CO₂ in air (Matheson Gas Products), sealed with Parafilm over a cotton plug, and incubated overnight in a Dubnoff shaker at 37°C. They were resuspended after 24 hours, and in some cases, 48 hours, as follows. The contents of the flasks were centrifuged at 600 g for 15 minutes and the supernatants saved. The cell clump was resuspended and reincubated as on the first day. The supernatant of the first centrifugation was then recentrifuged at 2000g for 30 minutes, and the supernate of this spin was stored at 4°C for 1-7 days or at -18°C for 1-3 weeks until it could be tested for pyrogenic activity.

B. Activation with Staphylococci. One flask was usually prepared as above with 0.5 ml. heat-killed *S. albus* added per dose.

C. Serum Studies. Suspensions were made up as above except that serum concentration was varied between 10-15% and 30-35% with patient and/or normal O- homologous serum.

D. Activation by lymphocytes. Spleen. Incubations with lymphocyte preparations were done using the cells eluted from the column as described. Incubation suspensions were made up as above with 10-15% serum in TCM to a total volume of 4-5 ml. per injected dose. $1-2 \times 10^8$ column cells were incubated alone as one lymphocyte dose, or with two leukocyte doses (at $3-5 \times 10^7$ WBC/dose). In some cases the leukocytes were added to the lymphocyte suspension at the outset, in others after lymphocytes had incubated alone for 24 hours.

Lymph node. One lymph node does (50-250 mg.) was used in place of spleen column lymphocytes. Otherwise the procedure was the same.

RESULTS

IA. Spontaneous endogenous pyrogen release by splenic tissue in vitro.

Thirteen spleen specimens from patients with Hodgkin's disease (HD) and twelve from patients with diseases other than Hodgkin's disease (non-Hodgkin's disease, NHD) were processed. The patients in the NHD group included six with idiopathic thrombocytopenic purpura (ITP), three with non-Hodgkin's lymphoma (NHL), one with autoimmune thrombocytopenia secondary to systemic lupus erythematosus, one with a splenic artery aneurysm, and one with a traumatic ruptured spleen.

The specimens were prepared and incubated as described in the Materials and Methods section. Briefly, the cells were suspended in tissue culture medium (TCM) with 10-15% serum and resuspended after 24 hours for a second 24 hours. Supernates from each incubation were tested for pyrogen by injection into rabbits. An example of a typical fever curve obtained is shown in Figure I. This has the characteristic short latency, early peak (< 90 minutes), and rapid fall to normal characteristic of EP fever. For each injection, peak temperature elevation occurring within 90 minutes was determined. Since the relation between pyrogen dose and temperature elevation is approximately

linear up to 0.8° C. elevation (84), the mean peak temperature elevation for any group of supernates was considered the measure of pyrogen for that group.

The results of comparison of pyrogen release by spleen cells from patients with various diseases is shown in Figure II. The mean peak temperature elevation produced by supernates from the first 24 hour incubation is $0.40 \pm .04^{\circ}$ C for the HD group, $0.13 \pm .02^{\circ}$ C for the NHL group, $0.20 \pm .04^{\circ}$ C for the ITP plus lupus group, and $0.39 \pm .10^{\circ}$ C for the other two. While results from the HD group and the last, and presumably most normal spleen group are similar, the last group is heavily weighted by the pyrogen production by cells from the ruptured spleen. This patient had been observed for two days preoperatively, and clinically demonstrated inflammation. Over all, comparing the HD group with the entire NHD group (Fig. 3), there is a significant difference ($p < .02$ by t test for difference of means) between the amount of pyrogen released during a first incubation from tissues in the two groups.

To look at this in a different way, the mean peak temperature elevation produced by injection of the supernate from the first incubation, was examined for each spleen; when the mean elevation was greater than 0.3° C, the tissue was considered "positive" for pyrogen release. Nine of the

thirteen spleens in the HD group were positive, and only three of twelve in the NHD group were positive. The number of cells and weight of tissue per dose are not significantly different in the two groups (Table I). Similarly the response to a phagocytic stimulus was not different, as all specimens in each group produced pyrogen in response to incubation with heat-killed staphylococci.

Differential counts were performed on a suspension from each spleen, and the percentage of pyrogen producing cells-polymorphonuclear leukocytes, eosinophils, and large monocytic cells-was compared. There were $27 \pm 2\%$ of these cells in the suspensions from the HD group, and $25 \pm 2\%$ in the suspensions from the NHD group. Thus there is no significant difference between the two groups with respect to the proportion of pyrogen producing cells.

The results of assay of spontaneous pyrogen release during the second 24 hours is also shown in Figures II and III. Pyrogen release was greater in all groups during the second period than during the first. The difference in pyrogen production between the HD group and the NHD group during this incubation is not significant. This result suggests that there has been a "warming up" to pyrogen release, a process in which the spleen cell suspensions from the HD patients had, for some reason, a head start. It

may also indicate a greater capacity for release, since the total production is therefore, greater in the HD group.

In the previous series of Bodel, spontaneous pyrogen release by spleen cells did not correlate with histological evidence of pathological involvement of the tissue. In this series only 20% of the spleens from patients with HD are positive for disease as determined by the surgical pathologist. This number is not large enough to allow division of the H D group into two subgroups (one with, and one without, clear-cut pathological involvement) for further analysis.

Figure I: Typical Fever Curve.

The temperature response in one rabbit to injection (at arrow) of supernate from incubation of one dose (3.5×10^8 cells) of spleen cells from a patient with Hodgkin's disease. The peak temperature elevation is 0.55°C .

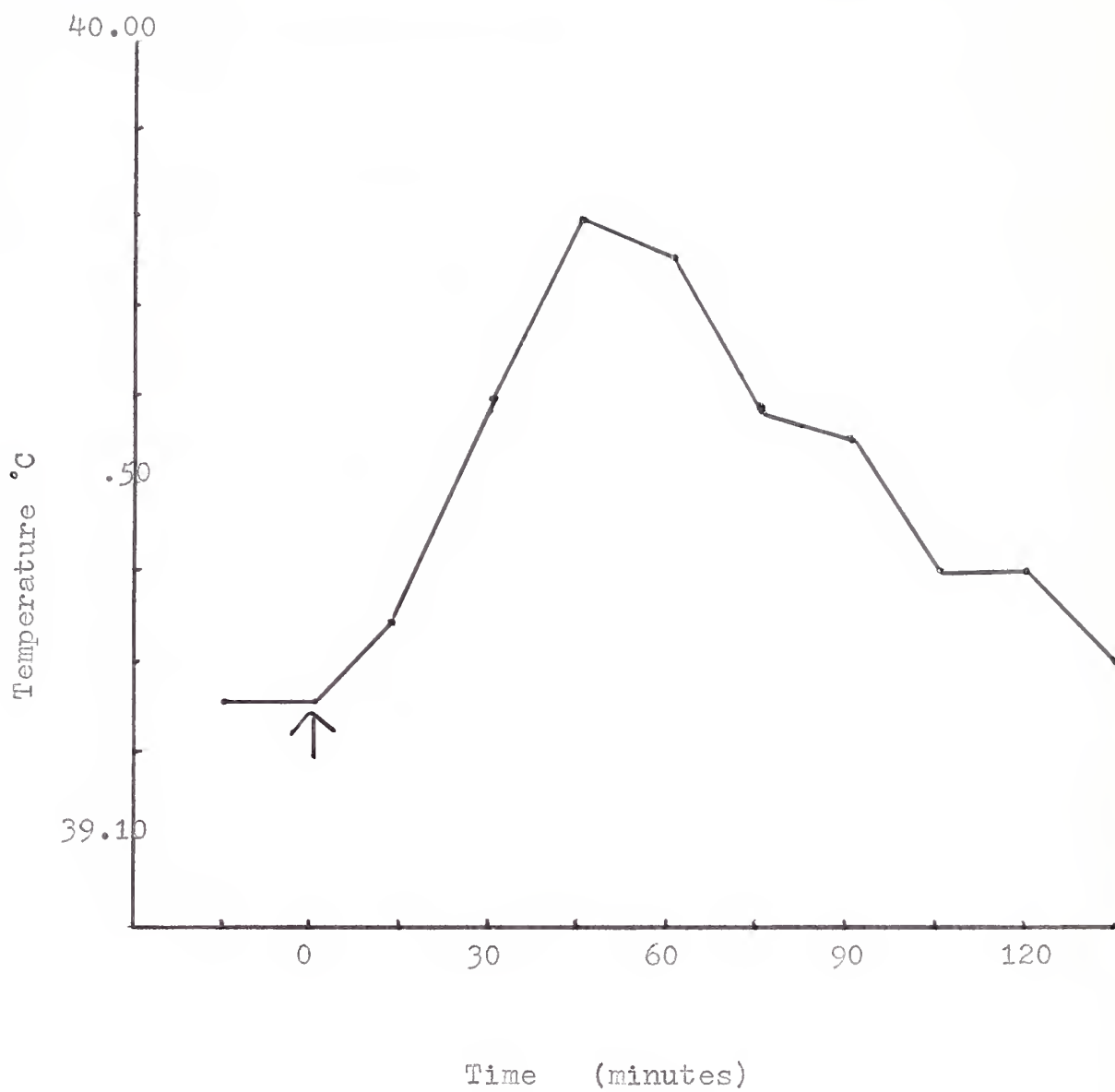


Figure 1



Figure II: Spontaneous Pyrogen Release by Spleen Cells
in vitro: I.

In this and subsequent figures, bars represent mean peak temperature elevations, \pm standard error of the mean, produced by injection of supernate from the incubation of tissue cells. In experiments with spleen, each dose of supernate, injected into a single rabbit, was derived from an average of 3.5×10^8 cells; in experiments with lymph nodes, from an average of 170 mg. (wet weight) of lymph node tissue. The numbers in parentheses refer to the number of supernate doses assayed in each group. The following abbreviations are used: HD, tissue from patients with Hodgkin's disease; NHL, tissue from patients with non-Hodgkin's lymphoma; ITP, tissue from patients with idiopathic thrombocytopenic purpura; SLE, tissue from patients with systemic lupus erythematosus. Day 1 refers to the first 24 hour incubation, Day 2, to the second. In this figure "other" refers to one tissue from a patient with traumatic ruptured spleen and one from a patient with a splenic artery aneurysm. Pyrogen release is presented for Day 1 and Day 2 incubations for the four different groups of spleen tissues.

Temperature Elevation °C

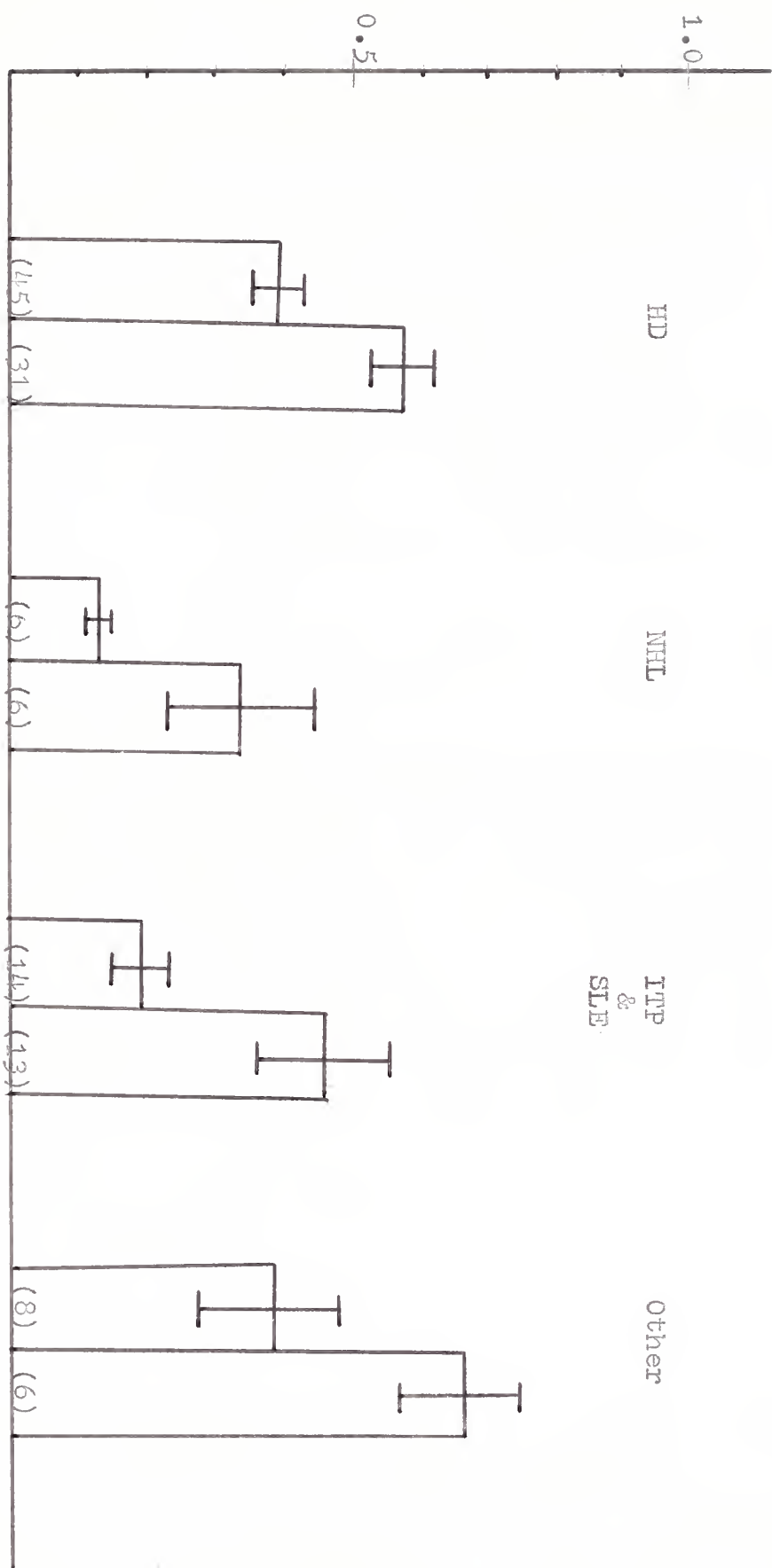


Figure II

Figure III: Spontaneous Pyrogen Release by Spleen Cells
in vitro: II.

In this and subsequent figures, NHD refers to tissue from patients with diseases other than Hodgkin's disease. In comparisons only differences significant to $p < 0.2$ by t test for means are noted. In this figure, mean peak temperature elevations following injection of supernates are compared for HD and NHD groups of tissues, during both a first and second incubation.

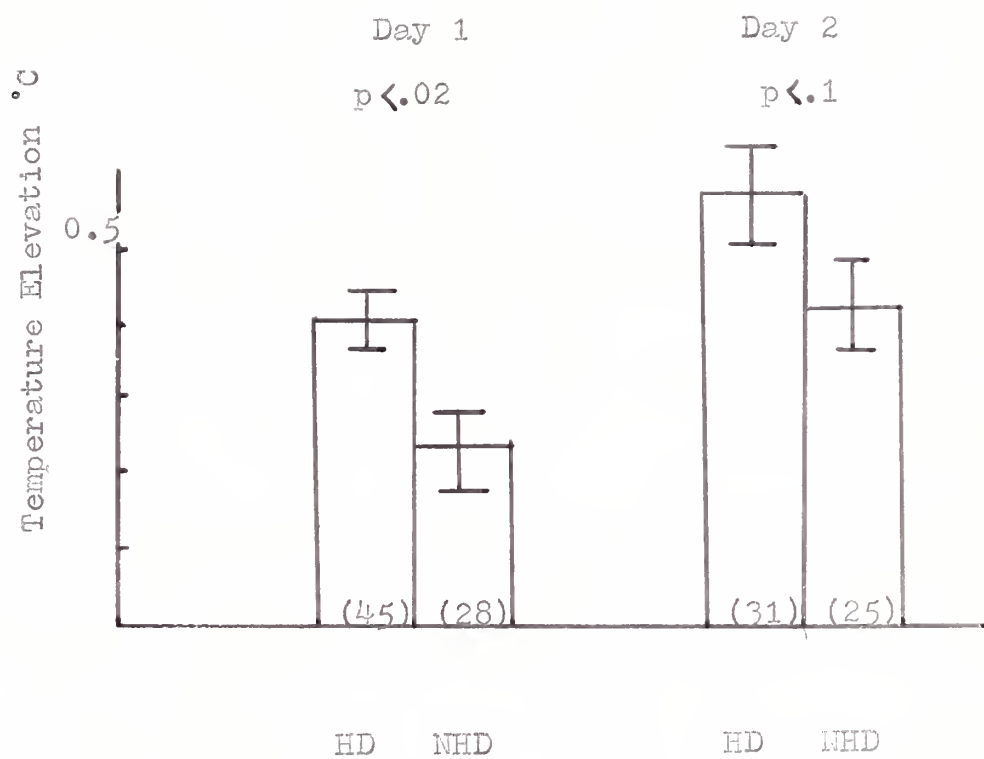


Figure III

Table I: Comparison of spleen cell populations between HD and NHD groups.

	HD	NHD
Wet weight(g) per dose	$1.04 \pm .07$	$1.01 \pm .06$
Free cells($\times 10^8$) per dose	$3.52 \pm .70$	$3.57 \pm .36$
Non-lymphocyte cells(%)	27 ± 2	25 ± 2

IB. Spontaneous endogenous pyrogen release by lymph node tissue in vitro.

Nodes were obtained from four patients with Hodgkin's disease, three patients with non-Hodgkin's lymphoma, three patients with metastatic carcinoma, and five with adenopathy with a reactive histology. This latter group included pathological diagnoses of lymphoid hyperplasia, sinus histiocytosis, and in one case, caseating granuloma consistent with lymphogranuloma venereum. The tissues were minced, and the cells incubated in TCM containing 10 or 30% serum for successive 24 hour periods, as described in Materials and Methods. Supernatants were injected into rabbits to determine the presence of endogenous pyrogen.

The results of pyrogen testing are shown in Figure IV. Clearly, only small amounts of pyrogen were released by cells from any of the groups, during a first or second incubation. The small release may be due to inadequate amounts of tissue per dose used in this system. The difficulty in obtaining larger lymph node specimens made this dose a necessity. There is no significant difference between pyrogen release by HD tissue compared with that by NHD tissue (Fig. V). Neither is there any significant difference between the two groups with respect to average amount of tissue per dose, being 171 ± 41 mg. in the HD

group, and 173 ± 21 in the NHD group. Estimates of cell types from differential smears were very difficult to perform in these experiments, because the cells appeared unusually fragile. However, the smears appeared comparable between the two groups. In only one case (a carcinoma node) were there more than 5% polymorphonuclear leukocytes in a smear.

Figure IV: Spontaneous Pyrogen Release by Lymph Node
Cells in vitro: I.

Pyrogen release is presented for Day 1 and Day 2 incubations for the four different groups of lymph node tissues. Reactive refers to lymph node tissue displaying a reactive histology (see text), Ca to tissue from patients with metastatic carcinoma.

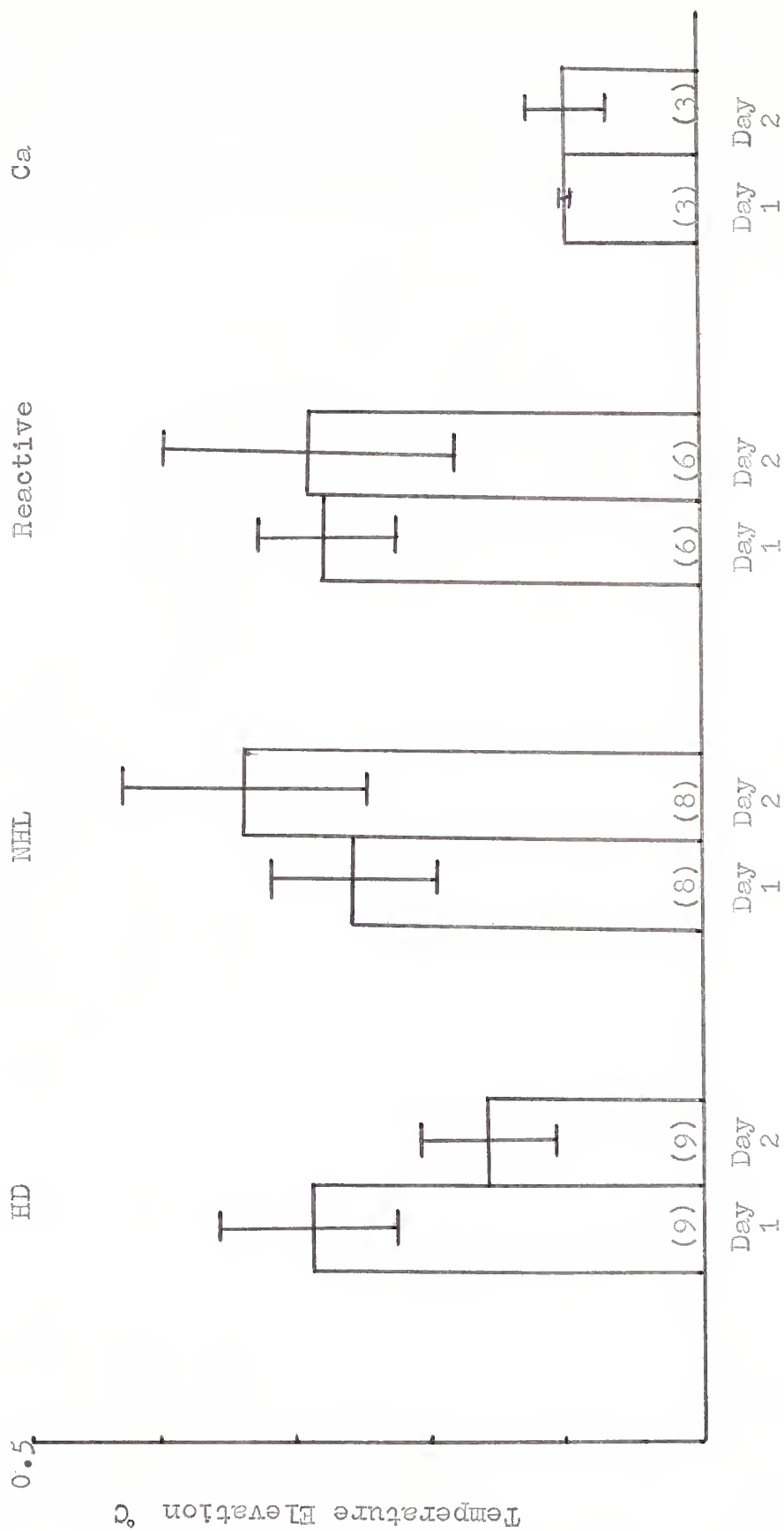


Figure IV

Figure V: Spontaneous Pyrogen Release by Lymph Node
Cells in vitro: II.

Mean peak temperature elevation following injection of supernates from incubations of lymph node from patients with Hodgkin's disease is compared with that from patients with other diseases. Data for both a first and second 24 hour incubation are shown.

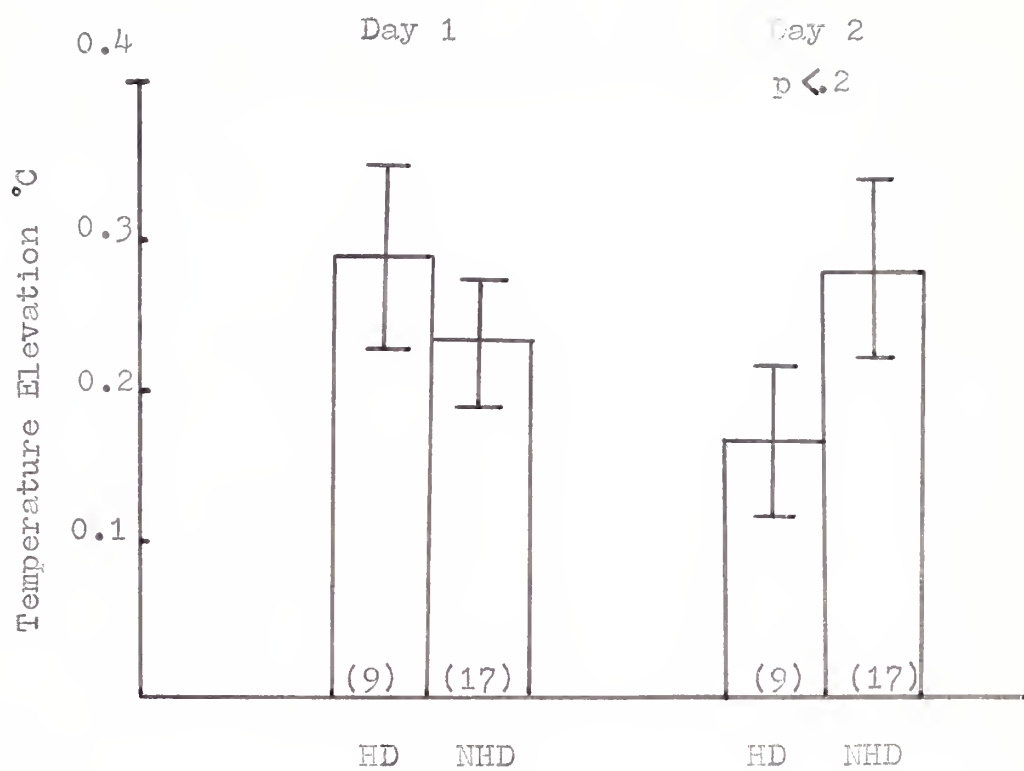


Figure V

IIA. Serum studies: the effect of varied serum concentration upon spontaneous EP release by lymphoid tissue in vitro.

Since, in Bodel's work, spontaneous endogenous pyrogen release has not been correlated with fever in the patient, it is possible that factors acting in vivo may modify the capacity of these cells for EP release. One of these factors may be a serum inhibitor. In the incubations in Bodel's series, the serum concentration in the incubation mixture was 10-15%. In order to investigate the possible effect of higher serum concentration on in vitro EP release, cells from lymph nodes and spleens were incubated in TCM with 10-15% serum and 30-35% serum. Serum was either autologous or homologous, from a normal O- donor, but for each individual specimen, the same serum was used at both concentrations. Incubation was carried out for two successive 24 hour periods.

Because of the small number of nodes (4) in this study, and the lack of significant difference between pyrogen release by the nodes during a first or second incubation (Fig. IV), pyrogenic responses to supernates from incubation on day one and day two were combined in determining an average. Therefore, Figure VI shows mean peak temperature elevations for HD and NHD tissues for both days.

Lymph node cells from Hodgkin's disease patients made less pyrogen when incubated with 30% serum than when incubated with 10% serum. However, because of the small number of supernates assayed, this apparent difference is not statistically significant ($p < .2$). There is no apparent, nor significant, difference between the serum concentrations in their effect on pyrogen release by NHD tissue.

The same study was performed with 11 samples of spleen tissue (Figure VII). Results for a first incubation alone, and for pyrogen release per incubation during two consecutive 24 hour incubations are presented. There is an apparent, but not statistically significant ($p < .2$), difference in pyrogen release during the first 24 hours incubation by spleen cells from HD patients, when incubated in the two concentrations of serum (Fig. VIIa). Cells incubated in 30% serum released less pyrogen than those incubated in 10% serum. There is no apparent effect of incubation in 30% serum as compared with 10% serum on pyrogen release by the NHD spleen cells.

When pyrogen release, per incubation, averaged over two incubations is examined, (Fig. VIIb), there is no significant difference evident between the effects of the two

serum concentrations on pyrogen release by spleen cells from either HD or NHD patients.

Since it was felt that autologous serum might be more effective than homologous serum in suppressing pyrogen release from HD tissues, a subseries (six specimens) was selected in which only those spleens which had incubated in both high and low concentrations of autologous serum were included (Figure VIIc, d). Again there is an apparent but not significant ($p < .2$) difference in pyrogen release during a first incubation when cells from HD spleens were incubated in 30% as compared to 10% serum. There is no significant effect of varying serum concentration demonstrated in the NHD group for the first incubation nor in either group if the pyrogen released on each of two incubations is averaged over both incubations.

Figure VI: Effect of Serum Concentration on Pyrogen
Release by Lymph Node Cells in vitro.

Comparison of mean peak temperature elevations following injection of supernates from incubation of lymph node tissue in either 10% or 30% serum, during a first or second 24 hour incubation. Data for both HD and NHD groups are shown.

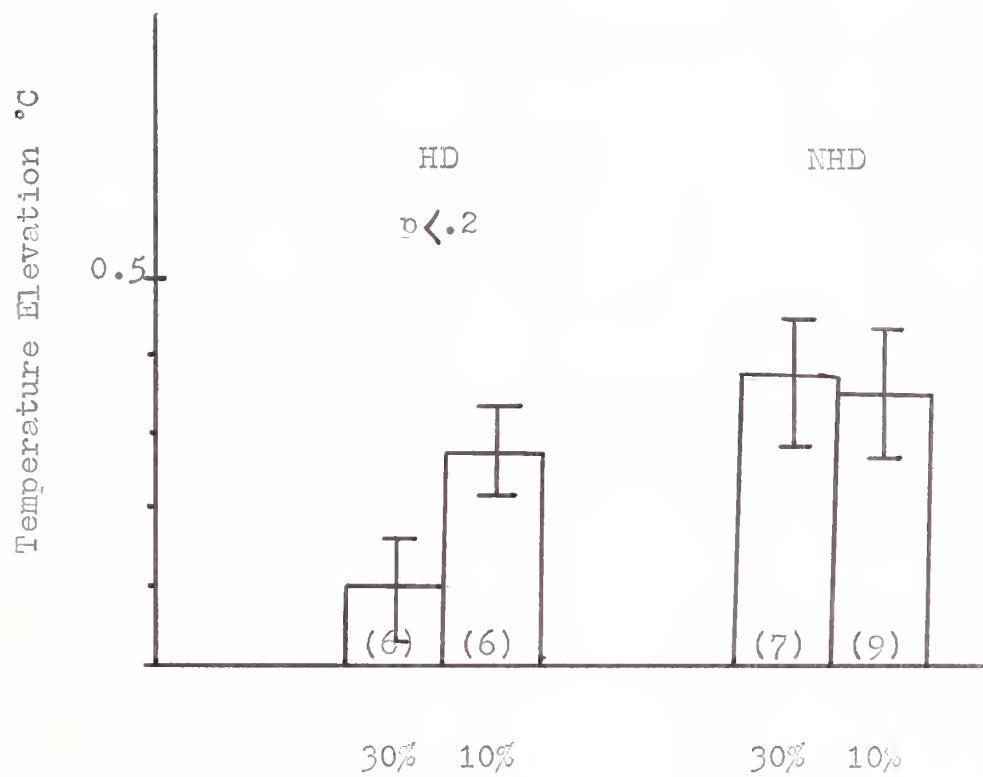


Figure VI

Figure VIIa: Effect of Serum Concentration on Pyrogen
Release by Spleen Cells in vitro: I.

Comparison of mean peak temperature elevations following injection of supernates from incubation of spleen cells in either 10% or 30% serum, during the first incubation. Data for both HD and NHD groups are shown.

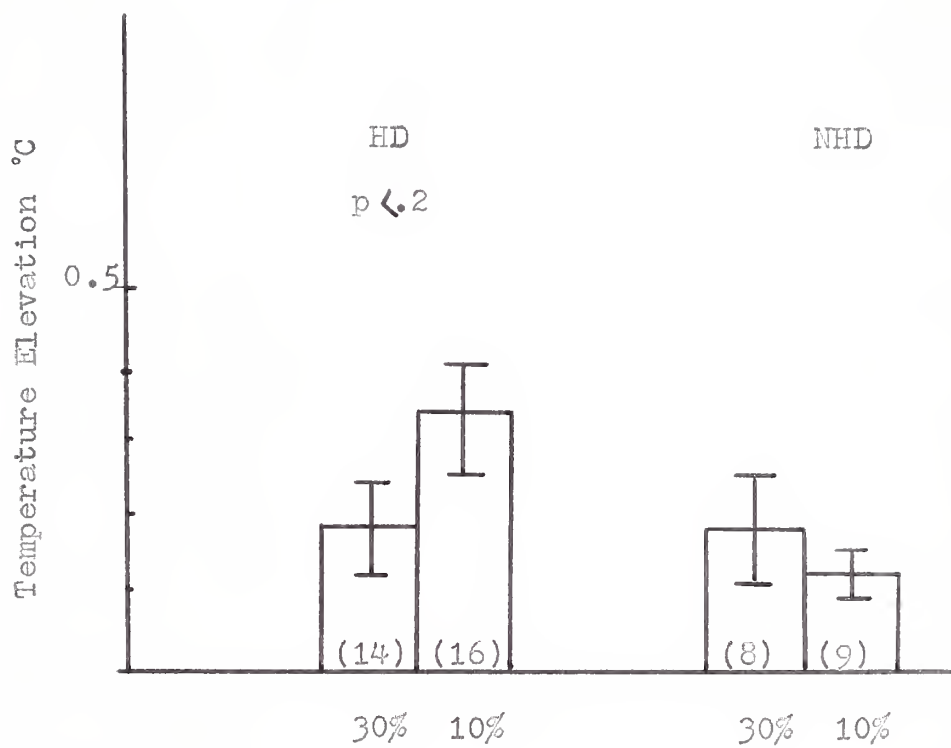


Figure VIIa

Figure VIIb: Effect of Serum Concentration on Pyrogen
Release by Spleen Cells in vitro: II.

Comparison of mean peak temperature elevations following injection of supernates from incubation of spleen cells in either 10% or 30% serum during either a first or second 24 hour incubation. Data are presented for both HD and NHD groups.

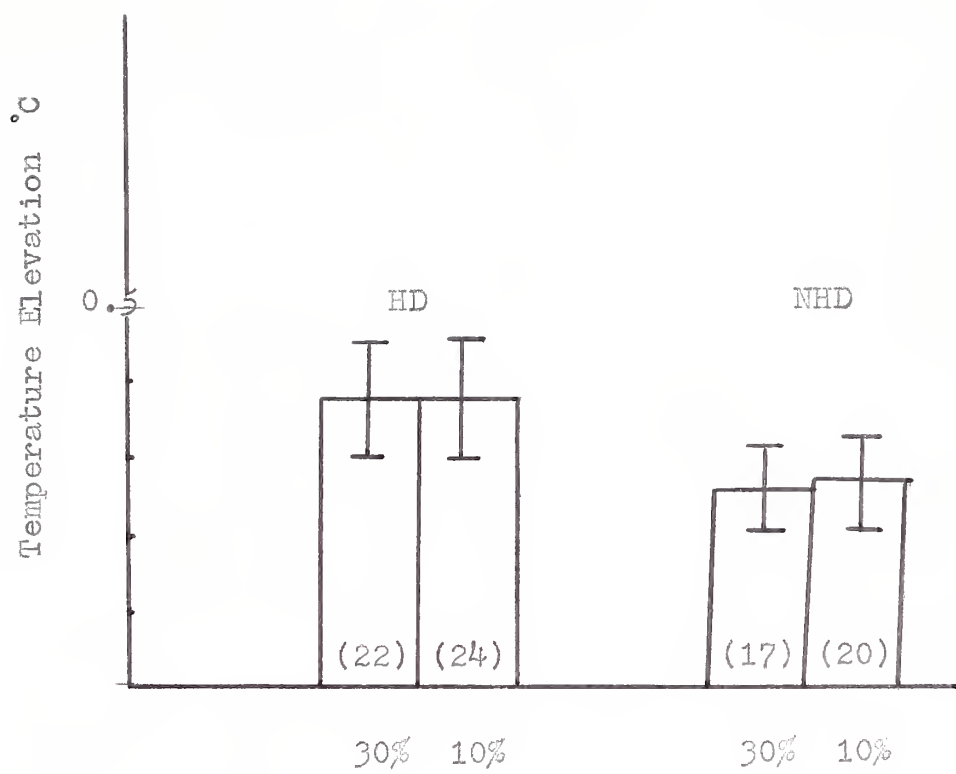


Figure VIIb

Figure VIIc: Effect of Serum Concentration on Pyrogen
Release by Spleen Cells in vitro: III.

Comparison of mean peak temperature elevations following injection of supernates from incubation of spleen cells in either 10% or 30% autologous serum during a first incubation. Data are presented for both HD and NHD groups.

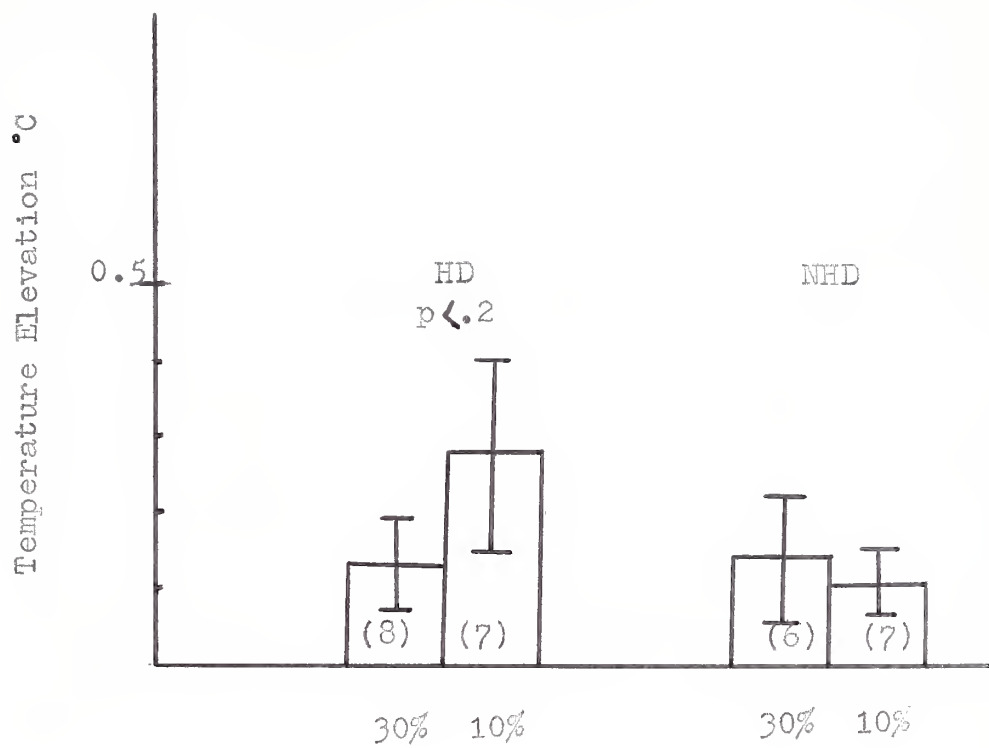


Figure VIIc

Figure VIId: Effect of Serum Concentration on Pyrogen
Release by Spleen Cells in vitro: IV.

Comparison of mean peak temperature elevations following injection of supernates from incubation of spleen cells in either 10% or 30% autologous serum, during either a first or second 24 hour incubation. Data are presented for both HD and NHD groups.

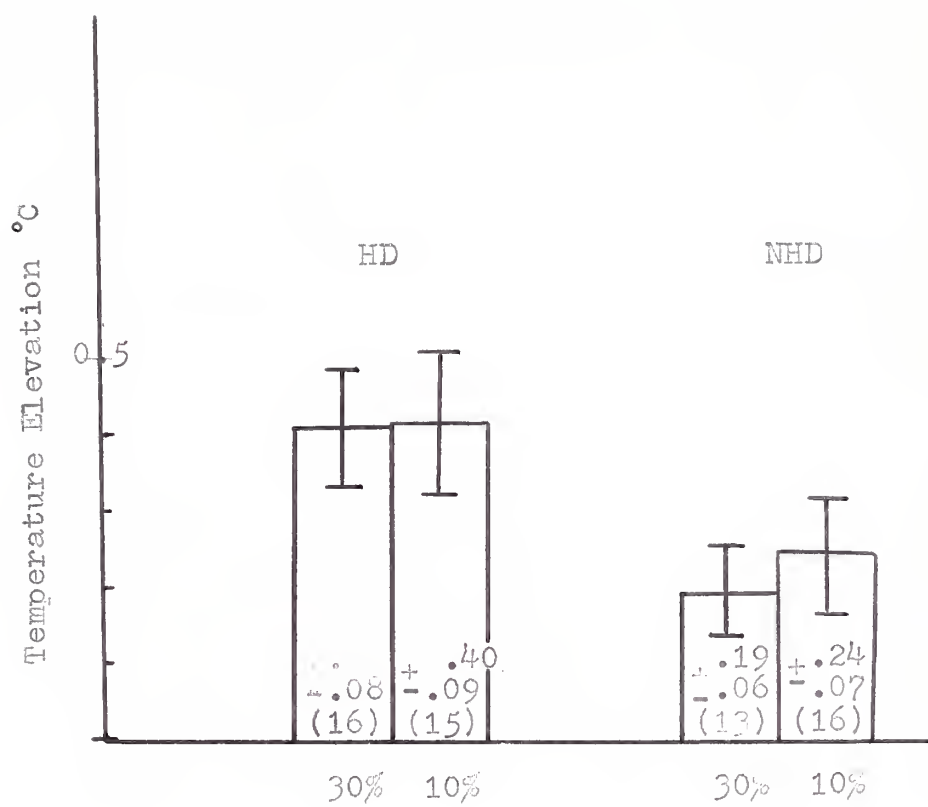


Figure VIId

IIB. Serum Studies: the effect of varied serum source upon spontaneous EP release by lymphoid tissue in vitro.

In order to further investigate the possibility that a factor in the serum of Hodgkin's disease patients may suppress pyrogen production, and thus, perhaps, be responsible for preventing EP release in vivo, aliquots of cells from a single tissue were incubated in autologous serum, or in homologous normal O- donor serum. Concentrations of 10-15% or 30-35% serum in TCM were both used, but in each autologous-homologous pair of incubations, the same concentration was employed.

Again, all incubations for each tissue over two days were combined in determining the average pyrogen released during each incubation of the seven lymph node specimens (Figure VIII). HD lymph node cells incubated with autologous serum released significantly less pyrogen per 24 hours of incubation, than did the same tissue incubated with homologous normal serum ($p < .05$). There is no significant difference between pyrogen release by lymph node cells in the NHD group when incubated with the two types of serum ($p < .2$).

In similar experiments with spleen cells from 16

specimens, varying the type of serum did not alter release of pyrogen during the first 24 hour incubation (Figure IXa) or release, per incubation, averaged over two consecutive 24 hour incubations (Figure IXb). Since, as noted in Figure VIIa, 30% serum appears to suppress release of pyrogen by spleen cells in the HD group, during the first 24 hours, an effect of autologous vs. homologous serum might be more marked in a group of tissues incubated at this concentration. Consequently results of eleven experiments in which spleen cells from either HD or NHD patients had been incubated in both 30% autologous and 30% homologous normal serum during the first 24 hour incubation were analyzed (Figure IXc). Spleen cells from patients with HD, but not from patients in the NHD group, produced less pyrogen when incubated with 30% autologous as compared to normal homologous serum, although the difference is not statistically significant ($p < .1$).

To look at these experimental results in a different way, comparison was made for each spleen cell preparation, between response to supernatant from incubation in 30%-autologous serum and in 30% homologous normal serum, during the same period. If the mean peak temperature elevations differed by greater than 0.15°C , the difference was presumed real. If the difference was less than 0.15°C , the responses

were considered equal. In experiments with six spleens from patients with HD, six comparisons could be made. In four, the incubation in homologous normal serum released more pyrogen; in two the responses were equal. In experiments with five spleens from NHD patients (2NHL, ZITP, 1 SLE) five comparisons could be made. In two cases more pyrogen was released by incubation in homologous serum; in one case by incubation in autologous serum. In two cases no difference was found.

Figure VIII: Effect of Serum Source on Pyrogen Release
by Lymph Node Cells in vitro.

Comparison of mean peak temperature elevations following injection of supernates from incubation of lymph node cells in either autologous (Auto) or normal homologous (Homo) serum, during either a first or second 24 hour incubation. Data are presented for both HD and NHD groups.

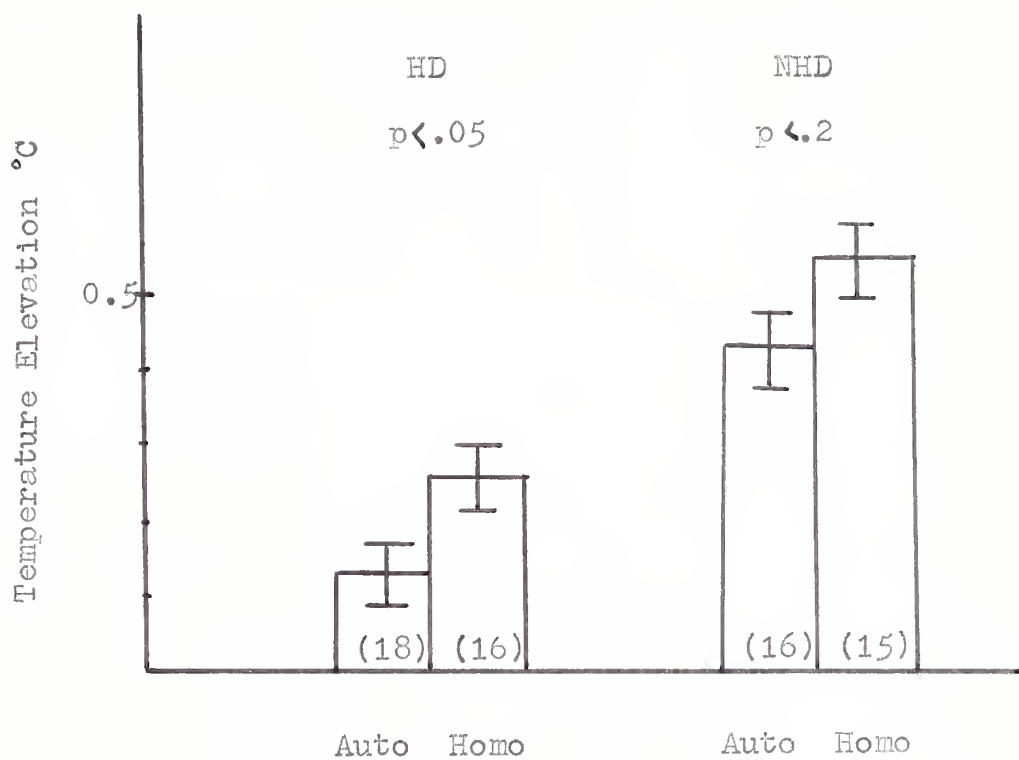


Figure VIII

Figure IXa: Effect of Serum Source on Pyrogen Release
by Spleen Cells in vitro: I.

Comparison of mean peak temperature elevations following injection of supernates from incubation of spleen cells in either autologous or normal homologous serum, during a first incubation. Data are presented for both HD and NHD groups.

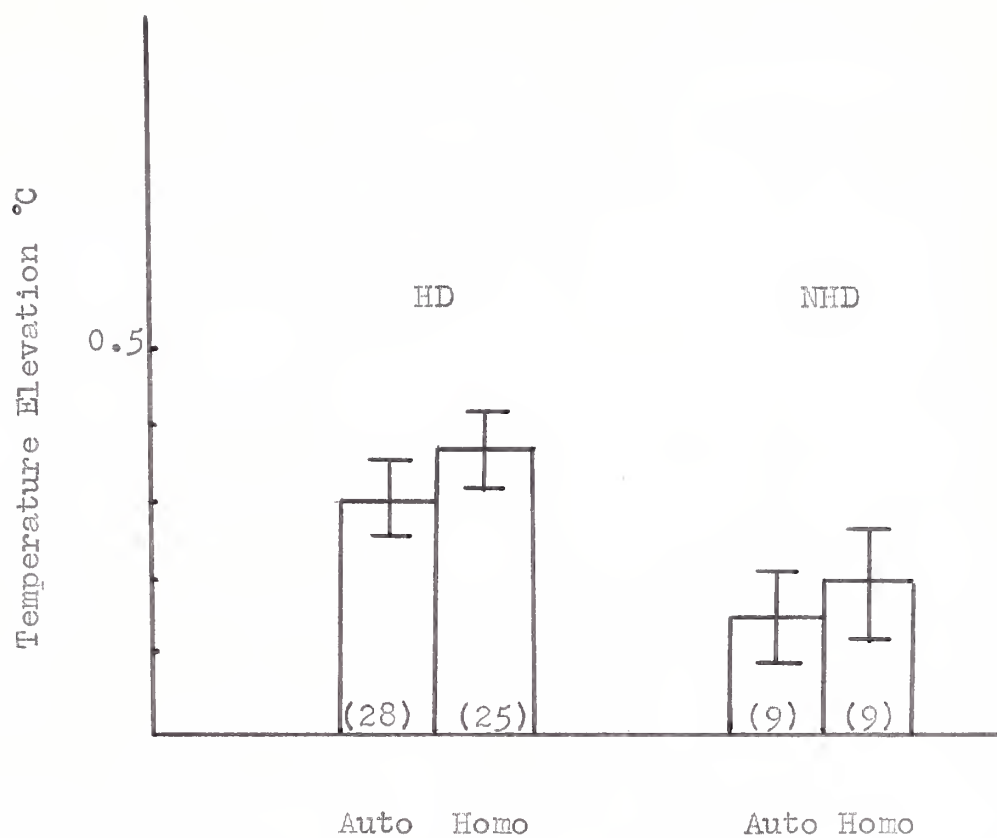


Figure IXa

Figure IXb: Effect of Serum Source on Pyrogen Release
by Spleen Cells in vitro: II.

Comparison of mean peak temperature elevations following injection of supernates from incubation of spleen cells in either autologous or normal homologous serum, during either a first or a second 24 hour incubation. Data are presented for both HD and NHD groups.

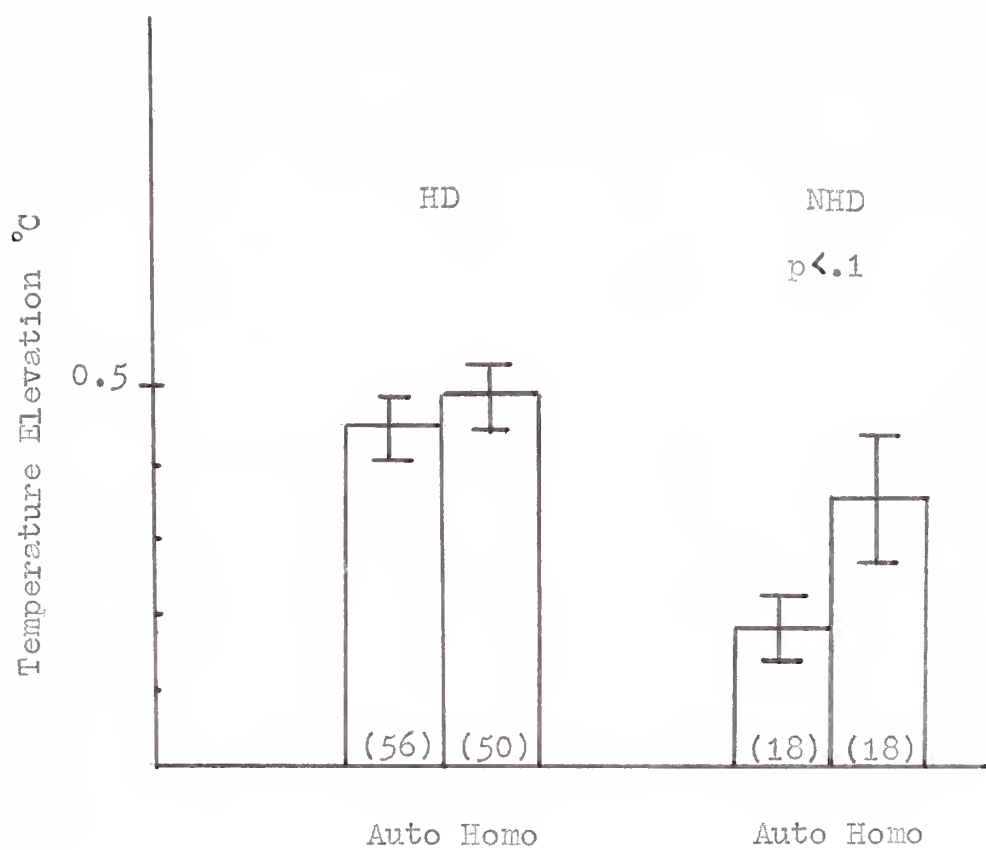


Figure IXb

Figure IXc: Effect of Serum Source on Pyrogen Release
by Spleen Cells in vitro: III.

Comparison of mean peak temperature elevations following injection of supernates from incubation of spleen cells in either 30% autologous or 30% normal homologous serum, during a first 24 hour incubation. Data are presented for both HD and NHD groups.

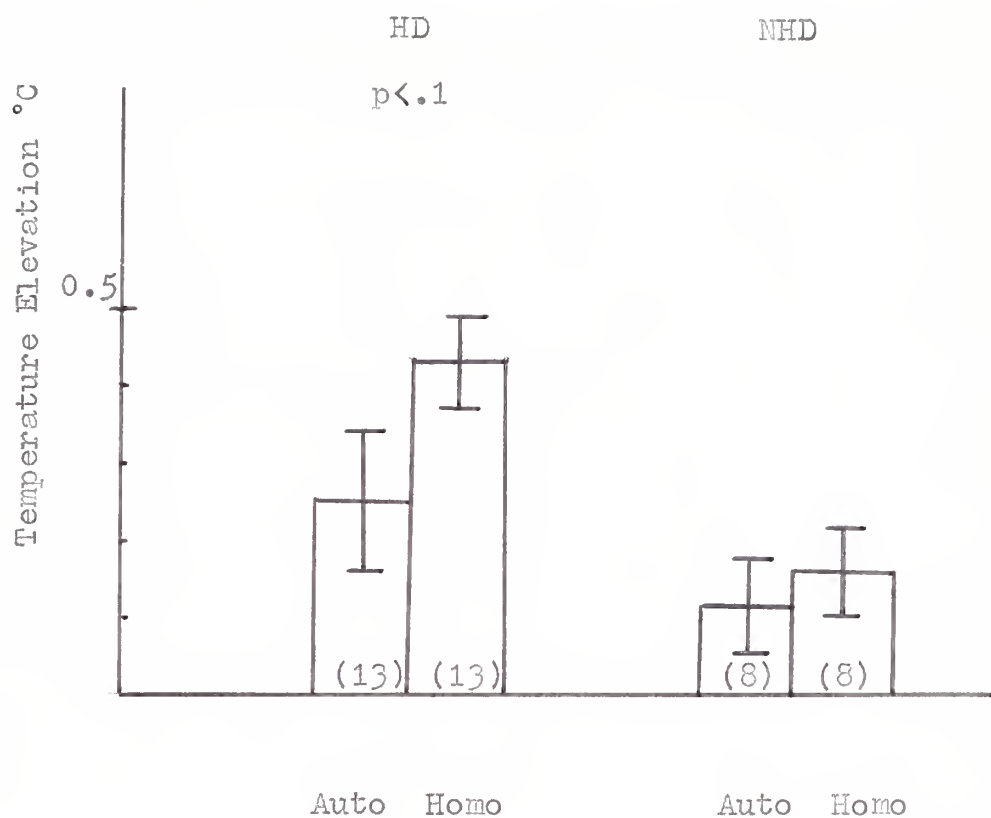


Figure IXc

III. Endogenous pyrogen release in vitro by a combined population of tissue lymphocytes and circulating blood leukocytes.

In order to examine the effect of lymphocytes from these tissues on induction of pyrogen release by cells capable of pyrogen release, but obtained in an inactive state, tissue lymphocytes were incubated together with blood leukocytes.

Since the available lymph node tissue was small in amount, and since its composition was, in all cases but one, under five per cent polymorphonuclear leukocytes, it was decided to use lymph node cells obtained directly from teased or minced lymph nodes as a lymphocyte source. To obtain spleen lymphocytes, a glass wool column was used, to which the phagocytic cells were allowed to stick (see Materials and Methods). The eluate cells were predominantly small lymphocytes (average of 89% in 17 experiments). The majority of the remaining 11% were large mononuclear cells, some of which may have been large lymphocytes rather than tissue monocytes, and, therefore, non-pyrogen producing.

Lymph node cells and spleen column eluate cells were then incubated with blood leukocytes from normal donors or from patients. Control suspensions of tissue lymphocytes alone, and of blood leukocytes, alone, were also incubated.

Supernates obtained after successive 24 hour incubations were assayed for pyrogen. A "positive" response required that the supernate from the incubation of the two cell populations together contain sufficient pyrogen to produce a mean peak temperature elevation greater than 0.3°C and greater than the mean peak temperature elevations of each of the control populations summed. When a supernate from an incubation containing either tissue lymphocytes or blood leukocytes alone produced sufficient pyrogen to produce a mean peak temperature elevation greater than 0.3°C , that experiment was excluded from the series, since induction of pyrogen release could not be determined in that case.

The results of the experiments with lymphocytes from spleen are presented in Table II. When spleens from HD patients were used, there were four positives in seven experiments. When the spleens were from patients in the NHD group, there was only one positive of 10. This single positive occurred with cells from the spleen that had ruptured two days preoperatively. The difference between the results in the two groups is significantly different by chi square ($p < .05$). There is no significant difference in the numbers of cells other than small lymphocytes between the two groups.

The observation that there is a significant difference between the activity of the two populations of lymphocytes suggests that the blood leukocytes were not responding to a

non-specific stimulus in these incubations. This possibility was further explored by incubating normal blood leukocytes with eluate from the column in the absence of spleen cells; no pyrogen release occurred. To further assure that the lymphocyte suspensions were not the source of the pyrogen, seven experiments were performed in which 1×10^8 cells from the column were incubated with staphylococci, to provide a phagocytic stimulus, and in no case did pyrogen release occur. Furthermore, a positive response was never obtained by injection of the supernate from the control incubation of the column lymphocytes alone.

When lymph node lymphocytes were studied in this system (Table III) there were three "positives" and no negatives in experiments using lymph nodes from patients with Hodgkin's disease. There were only 3 positives out of nine experiments using lymph nodes from patients in the other groups (two of four positive in the NHL group, one of one in the reactive group, none of four positive in the metastatic carcinoma group). The difference between the results in the HD and NHL groups is significant by chi square. ($p < 0.02$). There is no significant difference between the two groups with respect to percentage of cells other than small lymphocytes amongst the lymph node cells ($14 \pm 6\%$ compared with $24 \pm 18\%$). Two experiments were excluded, one each from the HD and NHL groups, because the lymph node cells produced

sufficient pyrogen, incubated alone, at the dosage used for incubation with blood leukocytes, to interfere with the interpretation of results from the combined incubation.

Table II: Pyrogen release in incubation mixtures containing spleen lymphocytes and blood leukocytes.

	Pyrogen Release		Total Experiments	Percent Positive
	Positive*	Negative		
HD	4	3	7	57
NHD	1	9	10	10

$p < .05$ by chi square

* Mean peak temperature elevation $\geq 0.3^{\circ}\text{C}$. See text for explanation.

Table III: Pyrogen release in incubation mixtures containing lymph node cells and blood leukocytes.

	Pyrogen Release		Total Experiments	Percent Positive
	Positive *	Negative		
HD	3	0	3	100
NHD	3	6	9	33

$p < .02$ by chi square

* Mean peak temperature elevation $\geq 0.3^{\circ}\text{C}$. See text for explanation.

DISCUSSION

These results, like those of the earlier series of Bodel (15, 16) show that spleen tissue from patients with Hodgkin's disease releases pyrogen spontaneously in vitro, and that this pyrogen release continues for at least 48 hours. When assayed in rabbits, the pyrogen behaves, like an endogenous pyrogen rather than endotoxin, since it causes a prompt, early fever with onset within fifteen minutes and peak temperature elevation within 90 minutes. Also, repeated injections of tissue pyrogen three times per day for four days, continues to cause undiminished pyrogenic responses.

Spleen cells from patients with Hodgkin's disease release significantly more pyrogen during a first 24 hour incubation than do spleen cells from patients with other conditions requiring splenectomy, including non-Hodgkin's lymphoma. By the second day, pyrogen release between the two groups is comparable.

Because pyrogen release continues for two days in vitro, and increases in amount over that period, it is likely that the pyrogen releasing cells are spleen monocytes. Polymorphonuclear leukocytes, unlike monocytes, release pyrogen only 12-16 hours after a single activating stimulus (17). However, it is possible that pyrogen release from this tissue may be effected by successive

activation of populations of polymorphonuclear leukocytes, each population active for only 12-16 hours.

It is unclear exactly what spontaneous endogenous pyrogen release in vitro represents, especially since pyrogen release by all groups of tissue was similar after 24 hours. It may indicate a previously altered state of the cells or it may result from non-specific activation by some process in the surgical or laboratory handling of the tissue. Clearly, if it is the latter, then there is some difference in the response of the HD and NHD groups to this activation stimulus. This difference could be one of activation threshold, degree of response to identical stimuli, or speed of response. If it represents a previously altered state of the cell, that alteration may be actual in vivo pyrogen release, there must be some in vivo modification of its effect, since none of the patients in the HD group were febrile at the time of, or shortly prior to surgery. This modification might result from EP inactivation in the circulation (such as by protein binding or enzymatic degradation), prevention of EP effect upon the thermoregulatory neurons of the hypothalamus, or prevention of fever by interruption of the efferent limb of the temperature regulating mechanism, such as by altered function of the autonomic nervous system.

The observed spontaneous EP release may also result from the combined effect of nonspecific activation and a

disease-specific difference in cell behavior between the two groups of tissues. For instance, release of pyrogen after the first 24 hours may be due to nonspecific activation, whereas the early response, seen in tissues of the HD group, may be due to a specific activating condition present in the one, but not the other group. The similar early release of EP from the spleen tissue of the patient with traumatic rupture and inflammation, a situation that seems likely to have led to in vivo cell activation, supports this conclusion.

Only 20% of the spleens from the HD group were "positive" for evidence of the disease on histological examination. This group was too small to allow division of the HD group into two subgroups (one with, and one without pathological involvement) for analysis. Previous work by Bodel, however, has shown no correlation between in vitro pyrogen release and histological involvement (16). This suggests that if the observed pyrogen release is, at least in part, disease specific, it either does not require pathological involvement of the tissue by the disease process, or it occurs in the tissue without leaving the histological evidence which we recognize as Hodgkin's disease. An example that such alterations take place is the demonstration of a HD associated antigen in pathologically "negative" spleens from patients with the disease (75).

The results of these experiments, then, suggest that, in Hodgkin's disease, there may be a relation between altered production of EP in vitro and a high frequency of fever in vivo. In a general way, they support the hypothesis that EP release from lymphoid tissue might be the mechanism for the fever of Hodgkin's disease. However, they do not explain the activation mechanism for EP release in patients with Hodgkin's disease.

Unlike the results in Bodel's series (16), there is no evidence here that lymph node tissue from patients with Hodgkin's disease releases significantly more pyrogen upon in vitro incubation than does nodal tissue from patients with other conditions requiring lymph node biopsy. This suggests that the lymph node is not a major site of pyrogen-producing cells in this disease. Further evidence for this conclusion is the finding that the pyrogen content of the supernates incubated with lymph node cells from two patients with febrile histories is not significantly different from that of supernates in the group as a whole (mean peak temperature elevation of 0.20°C compared with 0.29°C). This does not exclude a role for lymph node cells in the process of pyrogen production as activators of other cells. It probably does indicate that few cells capable of pyrogen production are present in the nodes. However, because the results of the serum studies, discussed below, show decreased

pyrogen release when lymph node cells from HD patients are incubated in autologous serum and in 30% serum, the capacity for EP release may have been masked, in this series, by the use of both autologous serum and 30% concentration in some incubations.

Studies of the effect of serum on pyrogen release were undertaken to look for factors which might modify the capacity of tissue cells for pyrogen release. Such a factor might help to explain the lack of correlation between in vitro EP release and clinical fever, reported in Bodel's series. Clearly, cells in vivo are exposed to the patient's serum at a concentration greater than the 10-15% used in our routine incubations. It was hypothesized that a factor might be present in the patient's serum which at most times prevents the cells' capacity for EP release from becoming manifest in vivo. Failure of this suppressive mechanism might then lead to a state of fever.

Two studies were set up, one in which serum concentration was either 10 or 30%, and the other in which the serum used was either autologous or normal homologous. In each study an effect of changes in serum was more apparent when lymph node, rather than spleen, tissue was used. This may be because their lesser pyrogen release is more easily inhibited. Alternatively, it may be that only pyrogen release by mononuclear cells is inhibited by a serum factor, and spleen pyrogen release may be due to pyrogen production by

both mononuclear and polymorphonuclear cells. However the results, in both tissues, consistently support the conclusion that incubation in 30% serum concentration, or autologous serum, leads to less pyrogen release by cells from Hodgkin's disease patients, as compared with incubation in 10% serum or with normal homologous serum. No such effect was observed on pyrogen release by cells from nodes or spleen of patients with non-Hodgkin's diseases. This finding suggests that the observed suppressive effect on tissue from HD patients is a disease-specific one.

Because the number of specimens is small and the differences are not all statistically significant, further studies will be required to establish this finding conclusively. However, these results fit with the hypothesis, outlined above, that a factor present in the serum of patients with HD suppresses pyrogen release by spleen and lymph node cells from these patients. Such a factor would be another example of host response to his tumor, others of which include blocking factor in malignant melanoma and an anti-blast transformation factor in Hodgkin's disease (86). Perhaps if the series could be extended, the results would become significant, or in the case of the serum concentration study, if even higher concentrations of serum were used, the effect might become more clear cut. The results are encouraging and suggest that further experiments should be done.

It is not clear however, why, when serum concentrations were varied, the suppressive effect of the higher serum concentration on spleen cells from patients with HD is not maintained when the results of incubation during each 24 hour period are considered together. This may reflect a difference in the means by which the HD spleen cells are being activated on the two days. As discussed above, spontaneous pyrogen release by the spleen cells may be partially in response to non-specific activation due to handling, and partly in response to disease-specific activating mechanisms. The serum factor may have an effect only on the disease-specific mechanism, presumed to be acting during the first day, and not on non-specific activation.

If the demonstration of spontaneous endogenous pyrogen release in vitro by spleen cells from Hodgkin's disease patients is not simply an artifact of handling, we must consider by what mechanism these cells have been altered in vivo. It is possible that, if the disease is caused by a virus, infection with the agent may activate the cell to pyrogen production. Parainfluenza virus is known to cause EP release from blood leukocytes in vitro, for instance (6). Another possible etiology for Hodgkin's disease, proposed by Vianna, et al, suggests that there is a virus infection in the barrier lymph nodes of the oropharynx or gut, which does not reach those lymph nodes which become what we recognize histologically as being involved by the disease, but

which seeds antigen-antibody complexes to the draining lymphoid tissue (98). The disease is presumed to be a response to the antigen-antibody complexes. Antigen-antibody complexes have also been shown to be activators of EP release in vivo and in vitro (85).

Another possibility is that the activator in these tissues is a lymphokine. In the hypothesized pathogenesis of Hodgkin's disease, proposed by Order and Hellman, and discussed previously, a cell-mediated immune response occurs in the involved tissue. Activated T lymphocytes are now known to produce a number of non-antibody products which are known as lymphokines (37). These include a mitogenic factor, a lymphocytotoxin, and a migration inhibiting factor for macrophages. There is some recent evidence that a lymphokine can activate blood cells to produce endogenous pyrogen.

In our experiments, lymphocytes from the spleens and lymph nodes were incubated together with circulating leukocytes to look for evidence of such a substance. In the seven incubations in which spleen lymphocytes from patients with Hodgkin's disease were combined with leukocytes, pyrogen release occurred in four instances. Similar release occurred in all three incubations in which lymphocytes from lymph nodes of patients with Hodgkin's disease were used. Both results are significantly different from those in which

lymphocytes from tissues of patients with other diseases were used, where pyrogen release occurred in only one of ten experiments using spleen cells and three of nine using lymph node cells.

These results provide evidence for some kind of inter-action between the two cell populations, which results in pyrogen release by one or both of them. The "lymphocyte population" was about 90% small lymphocytes, which are non-pyrogen producing cells; these preparations incubated alone or with a phagocytic stimulus, never released pyrogen. Thus even the 10% of cells which were possible pyrogen producing cells by microscopic examination were probably not pyrogen producing in these experiments. Consequently, it appears that the blood leukocytes are the source of the measured pyrogen. Since the same blood leukocytes did not release pyrogen when incubated under the same conditions without lymphocytes, it appears that some inter-action between the two cell populations was responsible for their activation.

Whether the important aspect of the inter-action is one specific to the action of lymphocytes from patients with Hodgkin's disease, or a non-disease-related action is unclear. Evidence against its being non-specific is the significant difference between the results of the experiments using lymphocytes from tissues of patients with HD and NHD.

Additional evidence against its being due to some artifact of the column procedure, in experiments with spleen tissue, such as wool particles which act as phagocytic stimuli to the leukocytes, is that an acellular column eluate, incubated with blood leukocytes, does not cause pyrogen release. Another non-disease-specific cell inter-action may be an immune inter-action between the two cell populations obtained from different individuals, in those cases in which the leukocytes were from a normal donor rather than from the patient himself. However, in the experiments with spleen cells, both positive and negative experiments, in both HD and NHD groups, contained patient lymphocytes and normal leukocytes. Thus, immune inter-action, even if present, cannot account for the difference between the results of experiments in which patient blood cells or normal blood cells were used. However, further studies of the mixed lymphocyte reaction will be needed to clearly resolve this question.

The evidence, at present, favors a disease-specific effect since induction of pyrogen release by blood leukocytes occurs more frequently when lymphocytes from HD patients are used. One explanation for a specific disease-related inter-action would be release of a lymphokine by altered lymphocytes present in HD patients. The results of these experiments do not however, prove such a conclusion. Further

supporting evidence would be the demonstration of pyrogen release by blood leukocytes after incubation with the supernate of a previous incubation of the lymphocytes alone.

Whether a lymphokine is, in fact, the mechanism for the demonstrated lymphocyte activity on blood leukocytes, spontaneous endogenous pyrogen release by spleen cells from Hodgkin's disease patients in vitro may result from the action of altered lymphocytes on tissue pyrogen-producing cells. This alteration may be due to a reaction to an antigen in the tissue. A Hodgkin's disease-associated antigen has been observed in both pathologically diseased and apparently normal spleens from patients with Hodgkin's disease (75, 77). Thus, the findings of altered lymphocyte function, demonstrated in this in vitro system, is consistent with, and would be required by, the hypothesis for the pathogenesis of Hodgkin's disease proposed by Order and Hellman.

SUMMARY

Lymph node and spleen cells from patients with Hodgkin's disease and various other disorders requiring splenectomy or lymph node biopsy were incubated in tissue culture medium in vitro. On the first day of incubation, spleen cells from patients with Hodgkin's disease released significantly more pyrogen than those from patients with non-Hodgkin's disease. Pyrogen release during a second 24 hour incubation was comparable in the two groups. There was no significant difference between the pyrogen release by the two groups of lymph nodes during either a first or second 24 hour incubation.

The effect of changing serum concentration in the incubation media from 10 to 30% was studied in this system. Lymph node cells from patients with Hodgkin's disease incubated in 30% serum released apparently, but not significantly, less pyrogen per day than the same cells incubated in 10% serum. Pyrogen release by cells from patients in the non-Hodgkin's disease group was not affected by the different serum concentrations. Pyrogen release during a first incubation, by spleen cells from patients with Hodgkin's disease was apparently less in 30% serum than in 10% serum, but this effect was not seen when results of both the first and second incubations were included together. No effect of

serum concentration was observed on spleen cells from patients with other diseases.

Different sera, either autologous or normal O-homologous serum, were compared for effect on pyrogen release in vitro. Lymph node cells from patients with Hodgkin's disease incubated in autologous serum released less pyrogen per day, over two days, than did lymph node cells from the same patients incubated in homologous O-serum. The different sera did not affect pyrogen release by lymph node cells from the non-Hodgkin's disease patients differently. Pyrogen release by spleen cells, whether from Hodgkin's or non-Hodgkin's patients, was not significantly different when homologous rather than autologous serum was used. In the experiments in which spleen cells from patients with Hodgkin's disease were incubated in 30% autologous or 30% homologous serum, cells incubated in autologous but not homologous serum, released apparently, but not significantly, less pyrogen during a first 24 hour incubation.

In the final group of experiments, spleen or lymph node lymphocytes were incubated with circulating blood leukocytes. Four of seven mixtures released pyrogen when lymphocytes from spleens of Hodgkin's disease patients were used. In only one of ten experiments was pyrogen released when spleen lymphocytes from patients with non-

Hodgkin's disease were used. The difference is significant. When lymph node cells were used, all three incubation mixtures containing cells from patients with Hodgkin's disease released pyrogen, whereas only three of nine released pyrogen when lymph node cells were from patients with non-Hodgkin's disease. The difference is significant.

Lymphoid tissue, from patients with Hodgkin's disease has been shown to be capable of spontaneous and prolonged endogenous pyrogen release in vitro. Some evidence has been found suggesting that serum factors may modify this capacity of tissue cells for EP release in vitro. Lymphocyte preparations from these tissues appeared to have the capacity to activate normal blood leukocytes to release pyrogen. These results suggest that the mechanism for the spontaneous release of EP by spleen preparations may involve the action of tissue lymphocytes on cells capable of pyrogen release, as by elaboration of a lymphokine. They further suggest that fever in Hodgkin's disease may result from the abnormal behavior of lymphoid tissue cells, as demonstrated in vitro.

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